# THE MICRO DETERMINATION OF TISSUE LIPIDS

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Recent interest in the nature and content of the lipids of tissues is constantly demanding more accurate methods for their determination. The present work was initiated as a result of the need for improved methods of estimating certain of the lipid fractions of glandular lipids in collaborative studies now under way between the departments of Physiological Chemistry and Anatomy (1).

A literature survey of extraction methods led us to the conclusion that complete lipid extraction can be attained by various methods and solvents. Obermer and Milton (2) and Rose and Riegel (3) reported that the 3 : 1 alcohol-ether solvent of Bloor and 1 : 1 alcohol-acetone of Schoenheimer and Sperry (4) are equally effective. Sperry (5) showed that extraction with these solvents is complete at room temperature for glandular tissues. We have confirmed these findings for beef liver and guinea-pig adrenal tissue, the former being relatively low and the latter high in lipid content.

A saponification method was developed from that of Bloor, using potassium hydroxide in alcohol-ether as described by Kelsey (6). Our method gave complete saponification of pure methyl esters of fatty acids, mixed triglycerides and pure cholesterol esters. Previous micro methods for the determination of fatty acids have generally been non-specific in nature, as, for example, the methods employing nephelometry, chylomicron count and dichromate oxidation. The titrimetric method of Stoddard and Drury (7) is specific as it measures the total carboxyl content after saponification and isolation of the fatty acids. It is a direct measure of the fatty acids provided their neutralization equivalent (mean molecular weight) is known. A simplified modification of the titrimetric method was developed and was tested against pure fatty acids.

The determination of cholesterol (both free and combined) was critically studied. Of the numerous methods proposed by other investigators, those based on the Liebermann-Burchard color reaction or the Windaus digitonin precipitation are the most widely accepted at present. The combination of the two principles by Kelsey (6) and by Schoenheimer and Sperry (4) has improved the accuracy and specificity of the analysis. These two methods were subjected to careful study. The Kelsey method in our hands gave erratic results even after considerable experience had been acquired in the use of the method. We could not use the Schoenheimer and Sperry procedure in its last detail because it required a specially designed colorimeter which was not available. A modification of their method was developed which can be used in an ordinary colorimeter and in the Evelyn Photoelectrometer, which we have employed. The method was found to give accurate results on both ester and free cholesterol.

In our experience we have found the phospholipid method of Bloor (8) to give satisfactory results in the determination of this lipid fraction.

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## EXPERIMENTAL

### I. Extraction of Tissues

Beef liver tissue was used in one series of studies of extraction methods. The tissue was ground up thoroughly and mixed well before the samples were weighed. Fifty ml. of 3 : 1 alcohol-ether was added to each sample and the extraction accomplished as indicated in Table I. The extracts were filtered into Erlenmeyer flasks and the extraction flasks washed with two 25 ml. portions of the solvent. The total extract was evaporated almost to dryness under reduced pressure and the residue extracted with petroleum ether. Total fatty acid and total cholesterol were determined on the petroleum ether extract by the method described later, the results appearing in Table I.

TABLE I

Analyses of Lipid Extracts of Beef Liver

| Method | Weight of Sample mg. | Fatty Acid | | Cholesterol | |
|---|---|---|---|---|---|
| | | % | Avg. | % | Avg. |
| Grind with sand, stand twelve hours with solvent | 1. 753.8 | 3.72 | ..... | 0.278 | ..... |
| | 2. 552.8 | 3.50 | 3.61 | 0.244 | 0.261 |
| Grind with sand, stand twelve hours at room temperature with solvent | 1. 661.2 | 3.77 | .... | 0.270 | ..... |
| | 2. 567.0 | 3.40 | 3.58 | 0.240 | 0.255 |
| Grind with sand, boil five minutes with solvent, stand twelve hours | 1. 721.0 | 3.56 | ..... | 0.277 | ..... |
| | 2. 616.6 | 3.46 | 3.51 | 0.252 | 0.264 |
| Grind with sand, reflux one hour, stand twelve hours | 1. 584.8 | 3.39 | ..... | 0.261 | ..... |
| | 2. 549.4 | 3.39 | 3.39 | 0.238 | 0.250 |
| Saponify tissue ninety minutes, ext. with solvent after acidification. | 1. 346.2 | 3.41 | ..... | 0.240 | ..... |
| | 2. 166.1 | 3.73 | 3.55 | 0.277 | 0.258 |

The average results in Table I indicate that essentially complete extractions were attained on liver tissue by the five procedures as described. Results by the saponification method serve as a standard of comparison.

Two of the procedures, described in Table I, were applied to a specimen of adrenal tissue of an old guinea pig. The two adrenals of this animal weighed 619 mg. The tissue was ground and mixed and four samples treated as described in Table II. This time, however, free and total cholesterol, phospholipid and total fatty acid were determined, the results serving to compare the efficiency of extraction by the two procedures.

Since the previous data showed complete extraction by standing for twelve hours at room temperature, this procedure was adopted. The details we have adopted for preparing a lipid extract follow.

*Extraction Procedure*

The fresh tissue sample was weighed by difference from a ground-glass stoppered weighing bottle into a mortar, mixed with sand, and ground up as described by Bloor (8). The mixture was transferred quantitatively to a 125 ml. Erlenmeyer flask. The mortar and pestle were washed with 1 ml. distilled water, and then with portions of the solvent. A total of 40 ml. 3 : 1 alcohol-ether (redistilled solvents) was added to the tissue. The flask was stoppered and allowed to stand

at room temperature overnight. The extract was then filtered through a fat-free filter paper into a 125 ml. Erlenmeyer flask. The residue on the filter was washed with two 15 ml. portions of fresh solvent and the washings added to the main extract. The combined extracts were evaporated almost to dryness under reduced pressure. A trap of the type used in the Van Slyke determination of nitrogen was convenient for this process. The flask was heated in a water bath placed on the steam bath. When a small aqueous residue remained in the flask, the trap and flask were disengaged from the suction tubing. From 7 to 9 ml. petroleum ether in three small portions was washed through the trap into the flask. The flask was then removed from the trap and stoppered. When a series of samples had reached this point, they were all extracted three times with petroleum ether. The petroleum ether was decanted into 15 ml. centrifuge tubes. The solvent was evaporated off before the second and third extractions of the residue were added to the tube. The substance thus extracted into the centrifuge tubes represented the total lipid and was used for the differential anlaysis of the lipid fractions.

TABLE II

LIPID ANALYSES OF GUINEA-PIG ADRENAL, FOLLOWING TWO EXTRACTION METHODS

| Method | Weight of Sample mg. | Phospho-lipid | | Free Cholesterol | | Total Cholesterol | | Fatty Acid | |
|---|---|---|---|---|---|---|---|---|---|
| | | % | Avg. | % | Avg. | % | Avg. | % | Avg. |
| Grind with sand, reflux one hour, stand twelve hours, room temperature | 116.7 | 3.07 | ..... | 0.21 | ..... | 1.18 | ..... | 14.58 | ..... |
| | 78.9 | 2.73 | 2.90 | 0.25 | 0.23 | 1.26 | 1.22 | 16.30 | 15.54 |
| Grind with sand, stand twelve hours, room temperature | 88.3 | 2.95 | .... | 0.27 | ..... | 1.27 | ..... | 15.50 | ..... |
| | 100.0 | 3.19 | 3.07 | 0.23 | 0.25 | 1.27 | 1.27 | 16.18 | 15.84 |

## II. THE SAPONIFICATION PROCEDURE

The efficiency of saponification by the Bloor method as described by Kelsey (6) was first tested on a specimen of pure cholesteryl palmitate, especially prepared for the purpose. The palmitic acid recovered from the saponification was estimated by the titration method, described in the next section of this paper. The results were erratic, the recovery of palmitic acid varying from 77 to 93 per cent on five samples. The method of saponification was therefore modified (see description below) and tested on a specimen of pure cholesteryl oleate. Preliminary tests on employing the method with a reaction time of 30, 60, and 90 minutes showed that even with this cholesteryl ester a thirty minute saponification is complete, recovery of the oleic acid being over 99 per cent.

*Description of Procedure*

The lipid to be saponified was dissolved in 5 ml. of 3 : 1 alcohol ether in a 15 ml. centrifuge tube, and 0.2 ml. of 30 per cent aqueous KOH added. If complete solution did not occur after vigorous stirring, alcohol up to 2 ml. was added. The tube was warmed in a beaker of hot water on a steam bath, the solution being allowed to boil gently for 30 minutes. Bumping was prevented by a boiling rod. The beaker was then placed on a hot plate and the solvent boiled off rapidly and completely. The tube was removed from the beaker and allowed to cool. To the aqueous residue was added just enough 1.8 N HCl to make the mixture slightly acid. The mixture was stirred vigorously and then 7 ml. petroleum ether added

and the mixture again stirred. The tube was allowed to stand about fifteen minutes, and then the petroleum ether solution was decanted from the water layer with the suction apparatus described by Bloor (8). The fatty acid solution was decanted into the 25 ml. Erlenmeyer flasks in which the titration was to be performed. The aqueous residue was re-extracted with two portions of petroleum ether and the extracts combined. The ether was evaporated off and the residue dissolved in alcohol and titrated.

In addition to the cholesteryl palmitate and oleate, previously mentioned, the procedure was tested on a specimen of mixed glycerides of olive oil (mean molecular weight of fatty acids 282), a specimen of brain lecithins, and a known synthetic mixture of all four types of lipids. The results are given in Table III.

TABLE III

Efficiency of Fatty Acid Recovery by Modified Saponification Procedure

| Fatty Acid in Sample mg. | Number of Determinations | Sample | Fatty Acid Recovered mg. | Recovery Avg. Per Cent |
|---|---|---|---|---|
| 5.39 | 3 | Methyl palmitate | 5.32 | 98.0 |
| 6.18 | 3 | Mixed triglycerides | 6.16 | 100.2 |
| 8.40 | 3 | Lecithins | 8.31 | 99.0* |
| 4.02 | 3 | Cholesterol oleate | 4.00 | 99.4 |
| 10.00 | 4 | Mixed esters (above four) | 10.05 | 100.4† |

*Range of recoveries 98.2–100.4%.
†Range 100.1–101.2%.

## III. A Titrimetric Method for the Determination of Fatty Acids

The fatty acids employed in this work were highly purified specimens, prepared especially for solubility determinations by H. F. Foreman (9) in this laboratory.

In preliminary work the oxidative method of Bloor (10), applied to oleic acid, was found to give satisfactory results, recoveries on 5 mg. samples being 96 to 102 per cent. The titration method of Stoddard and Drury (7) also proved satisfactory. It was our experience, however, that phenolphthalein possessed some advantages over thymolphthalein. This latter indicator goes through a series of color changes from colorless to yellow to green to blue in alcoholic solution, thus making the end-point difficult to determine. Our titrations with 0.035 N alkali were carried out with a special burette. The details of the method and results follow.

### *Description of Titrimetric Method*

An automatic 5 ml. Exax burette, graduated to hundredths, was fitted with a special tip which delivers 150 drops per ml. solution. The standard alkali was kept in a paraffin coated bottle protected from the carbon dioxide of the air by Ascarite and concentrated alkali traps. The sample of fatty acid, usually in petroleum ether solution, was measured into a 25 ml. Erlenmeyer flask and the solvent evaporated off. The residue was dissolved in 10 ml. 95 per cent alcohol, measured by a pipette. From 5 to 7 drops of 1 per cent phenolphthalein was added, and the sample titrated to the first faint pink color. A series of blank titrations on 10 ml. samples of the alcohol was made and the titrations corrected accordingly. The blank amounted to 0.063 ml. The alkali used in the titration was standardized against Bureau of Standards potassium acid phthalate. Results on pure oleic, palmitic and stearic acids are shown in Table IV.

TABLE IV

THE TITRIMETRIC DETERMINATION OF PURE FATTY ACIDS

| Specimen | No. of Det'ns | Alkali ml. (Corrected) | Fatty Acid | | Recovery Per Cent |
|---|---|---|---|---|---|
| | | | Taken mg. | Found mg. | |
| Alcohol Blank | 5 | 0.063 | ..... | ..... | ... |
| Stearic Acid | 5 | 1.173 | 11.30 | 11.31 | 100 |
| " " | 3 | 0.581 | 5.65 | 5.60 | 99 |
| " " | 2 | 0.459 | 4.52 | 4.43 | 98 |
| " " | 3 | 0.351 | 3.39 | 3.35 | 99 |
| " " | 2 | 0.236 | 2.26 | 2.27 | 100 |
| " " | 3 | 0.118 | 1.13 | 1.14 | 101 |
| Palmitic " | 2 | 1.240 | 10.76 | 10.76 | 100 |
| " " | 3 | 0.613 | 5.38 | 5.31 | 99 |
| " " | 3 | 0.381 | 3.23 | 3.30 | 102 |
| Oleic " | 1 | 1.171 | 11.30 | 11.19 | 99 |
| " " | 2 | 0.353 | 3.39 | 3.38 | 100 |

## IV. AN INVESTIGATION OF FREE AND COMBINED CHOLESTEROL PROCEDURES

Pure samples of cholesterol were prepared from beef brain tissue. Standard stock solutions of this cholesterol were made up in petroleum ether and kept tightly stoppered at 0° C. Samples were measured from these stock solutions for all subsequent analyses on pure cholesterol.

The Evelyn photoelectrometer (11) was used throughout the investigation for the measurement of the color intensity of the cholesterol samples. The galvanometer reading, G, when calculated to L, or (2-log G), should give a straight line of slope K when plotted against the weight in mg. of cholesterol in the samples.

The accuracy of the color determination as recommended by Kelsey was first studied. The samples were measured from the stock solutions into 25 x 200 mm. soft glass test tubes which had been optically tested for use in the photoelectrometer. The solvent was evaporated off, and 10 ml. chloroform, 1.0 ml. acetic anhydride, and 0.1 ml. concentrated sulfuric acid were added to each tube. The samples were well stoppered, mixed, kept at a temperature of 23° C. for twenty minutes and read in the instrument. The average values for K found for two series of twenty samples from 0.1 to 1.0 mg. in size, were $0.68 \pm 0.054$ and $0.71 \pm 0.061$. The variation is over 8 per cent.

Kelsey's method of separating free and ester cholesterol by digitonin precipitation was next studied. It involves the evaporation to dryness of a mixture of the lipid and a solution of alcoholic digitonin. Extraction of the residue with petroleum ether removes the cholesterol ester and other lipids. The cholesterol digitonide is decomposed by refluxing it in benzene. The addition of petroleum ether to the hot benzene dissolves the cholesterol and the digitonin is precipitated. The variable results by this method are shown in Table V.

A further study of the method was made on samples of pure cholesterol, in which the amount of cholesterol not precipitated by the digitonin, the amount not decomposed by the benzene, and the amount normally determined by the Kelsey method were determined. These three fractions were determined by our modified method described below. The comparatively large samples were used in order to establish the limits of the method when 5 ml. 0.2 per cent digitonin solution was used. The results are shown in Table VI.

In the Schoenheimer and Sperry method (4) the cholesterol is quantitatively precipitated as the digitonide in a mixture of equal parts of absolute alcohol,

acetone, and water. The washed and dried precipitate is then dissolved in 1 ml. of hot glacial acetic acid and the solution is cooled. Two ml. of acetic anhydride and 0.1 ml. of concentrated sulfuric acid are added and the color intensity of the solution determined after twenty-five minutes at 25° C. In order to use the

TABLE V

THE DETERMINATION OF FREE AND ESTER CHOLESTEROL IN KNOWN SYNTHETIC MIXTURES BY THE KELSEY METHOD

| Composition of Sample | Recovery of Free Cholesterol | | Recovery of Ester Cholesterol | |
|---|---|---|---|---|
| | mg. | Per Cent | mg. | Per Cent |
| 0.491 mg. free, 0.491 mg. ester | 0.478 | 97 | 0.540 | 110 |
| 0.491 mg. free, 0.0 mg. ester | 0.468 | 96 | 0.064 | ... |
| 0.0 mg. free, 0.491 mg. ester | 0.087 | ... | 0.469 | 96 |
| 0.261 mg. free, 0.0 mg. ester | 0.228 | 88 | ... | ... |
| 0.261 mg. free, 0.491 mg. ester | 0.235 | 90 | ... | ... |
| 0.261 mg. free, 0.786 mg. ester | 0.269 | 103 | ... | ... |
| 0.261 mg. free, 0.982 mg. ester | 0.284 | 109 | ... | ... |
| 0.417 mg. free, 0.0 mg. ester | 0.383 | 92 | ... | ... |
| 0.823 mg. free, 0.0 mg. ester | 0.711 | 86 | ... | ... |
| 0.0 mg. free, 0.491 mg. ester | 0.019 | ... | ... | ... |
| 0.0 mg. free, 0.786 mg. ester | 0.019 | ... | ... | ... |

TABLE VI

CHOLESTEROL DETERMINATION BY KELSEY METHOD

| Cholesterol in Sample | Cholesterol Not Precipitated | | Cholesterol In Residue | | Cholesterol Determined | |
|---|---|---|---|---|---|---|
| mg. | mg. | Per Cent | mg. | Per Cent | mg. | Per Cent |
| 0.166 | 0.005 | 3.0 | 0.005 | 3.0 | 0.141 | 84.8 |
| 0.333 | 0.027 | 8.2 | 0.012 | 3.0 | 0.288 | 86.4 |
| 0.498 | 0.065 | 13.2 | 0.012 | 2.5 | 0.414 | 83.0 |
| 0.834 | 0.000 | 0.0 | 0.034 | 4.1 | 0.789 | 94.6 |
| 1.668 | 0.108 | 6.5 | 0.311 | 18.6 | 1.227 | 73.5 |
| 2.502 | 0.006 | 0.2 | 0.852 | 34.0 | 1.643 | 65.7 |
| 3.336 | 0.539 | 16.2 | 1.051 | 31.4 | 1.762 | 52.8 |
| 4.270 | 1.386 | 32.5 | 1.106 | 25.9 | 1.864 | 43.7 |
| 5.002 | 2.080 | 41.6 | 0.754 | 15.1 | 2.160 | 43.2 |

Evelyn photoelectrometer in our work, all the reagents were doubled and the color intensity read through the 6 ml. aperture. On samples of cholesterol from 0.1 to 0.4 mg., the value of K was found to be $1.04 \pm 0.04$. Corrected to a volume of 11 ml. for comparison with the value of $0.70 \pm .06$ obtained by the Kelsey method, K becomes $0.57 \pm 0.02$. This lower value for K explains why small samples of cholesterol are more difficultly determined using acetic acid as the solvent.

Investigation revealed that the cholesterol digitonide could be dissolved in a relatively small volume of hot glacial acetic acid. To this solution, chloroform could be added without causing the precipitation of either the cholesterol digitonide or free digitonin. The color intensity of this solution approached that of a chloroform solution of cholesterol and could easily be read in the photoelectrometer even in 12 ml. volumes.

Experiments were conducted to ascertain the most effective concentration of each reagent, glacial acetic acid, chloroform, acetic anhydride, and sulfuric acid, as well as the most desirable temperature for the color reaction. The results are shown in Table VII.

TABLE VII

THE LIEBERMANN-BURCHARD COLOR REACTION: EFFECT OF TEMPERATURE AND CONCENTRATION OF REAGENTS

| Sample | No. of Samples | Light Filter | ml. | | | | Temp. | K | Time |
|---|---|---|---|---|---|---|---|---|---|
| | | | Acetic Acid | Chloroform | Anhydride | Sulfuric Acid | | | |
| Cholesterol | 40 | 6600 | 0 | 10 | 1 | 0.1 | 23 | 0.70 | 20 |
| Cholesterol | 5 | 6600 | 0 | 10 | 1 | 0.1 | 25 | 0.73 | 25 |
| Cholesterol | 5 | 6600 | 0 | 10 | 1 | 0.1 | 17 | 0.59 | 65 |
| Cholesterol | 5 | 6200 | 4 | 0 | 8 | 0.4 | 26 | 0.65 | 30 |
| Cholesterol | 5 | 6200 | 4 | 0 | 8 | 0.4 | 27 | 0.65 | 35 |
| Cholesterol | 5 | 6200 | 4 | 0 | 8 | 0.4 | 18 | 0.57 | 65 |
| Cholesterol | 5 | 6200 | 2 | 8 | 2 | 0.5 | 32 | 0.83 | 12 |
| Cholesterol | 6 | 6200 | 2 | 8 | 2 | 0.5 | 16 | 0.81 | 50 |
| Digitonide | 5 | 6200 | 4 | 0 | 8 | 0.4 | 18 | 0.52 | 75 |
| Digitonide | 1 | 6200 | 2 | 0 | 10 | 0.5 | 18 | 0.66 | .. |
| Digitonide | 1 | 6200 | 6 | 0 | 6 | 0.5 | 18 | 0.53 | .. |
| Digitonide | 1 | 6200 | 2 | 3 | 7 | 0.5 | 18 | 0.70 | .. |
| Digitonide | 1 | 6200 | 2 | 4 | 6 | 0.5 | 18 | 0.68 | .. |
| Digitonide | 1 | 6200 | 2 | 5 | 5 | 0.5 | 18 | 0.74 | .. |
| Digitonide | 1 | 6200 | 2 | 6 | 4 | 0.5 | 18 | 0.75 | .. |
| Digitonide | 1 | 6200 | 2 | 8 | 2 | 0.5 | 18 | 0.73 | .. |
| Digitonide | 1 | 6200 | 2 | 9 | 1 | 0.5 | 18 | 0.66 | .. |
| Digitonide | 1 | 6200 | 2 | 8 | 2 | 0.2 | 18 | 0.45 | 110 |
| Digitonide | 1 | 6200 | 2 | 8 | 2 | 0.4 | 18 | 0.73 | 65 |
| Digitonide | 2 | 6200 | 2 | 8 | 2 | 0.5 | 18 | 0.72 | 60 |
| Digitonide | 1 | 6200 | 2 | 8 | 2 | 0.7 | 18 | 0.70 | 45 |

The precipitation of the cholesterol as the digitonide by the Schoenheimer and Sperry method was incomplete for samples of 0.1 to 0.5 mg. This is indicated in Table III, where the digitonide gives consistently lower values for K than the pure cholesterol samples after comparable treatment. Increasing the volume of 0.2 per cent aqueous digitonin solution from one to four ml. per sample did not result in the quantitative precipitation of the cholesterol. However, by using 2 ml. of 0.4 per cent aqueous digitonin, 2 ml. acetone, and 2 ml. alcohol and carrying out the precipitation at a lower temperature, quantitative results were obtained as shown in Table VIII.

TABLE VIII

THE PRECIPITATION OF CHOLESTEROL DIGITONIDE

| Volume of Digitonin Solution ml. | Temperature ° C. | L | Cholesterol Calculated mg. | Cholesterol Recovery Per Cent |
|---|---|---|---|---|
| 0 | 26 | 0.260 | 0.321 | 98 |
| 6 | 26 | 0.254 | 0.314 | 96 |
| 6 | 16 | 0.260 | 0.321 | 98 |
| 6 | 8 | 0.260 | 0.321 | 98 |

*Description of Cholesterol Procedure*

To the lipid sample in a 15 ml. centrifuge tube was added 2 ml. each of acetone, alcohol and 0.4 per cent digitonin solution (prepared by evaporating a solution of one gram digitonin in one liter distilled water to 250 ml.). The mixture was stirred thoroughly and the tube was stoppered and placed in the refrigerator overnight. The tube was then centrifuged at a high rate of speed for ten minutes and the supernatant liquid decanted. The precipitate was washed with acetone-ether and then with ether as described by Schoenheimer and Sperry. The precipitate was dried by placing the tube in a beaker of warm water. The tube was then placed in a pan containing about two inches of sand and kept in an oven at about 120° for about twenty minutes. Then the pan and its contents were removed. Two ml. of glacial acetic was pipetted into the tube and the contents of the tube well mixed. The tube was removed from the sand bath after a few minutes and allowed to come to room temperature. Ten ml. of the color reagent was pipetted into the sample. The reagent was prepared by mixing 20 ml. acetic anhydride (C. P. 99 per cent), 80 ml. chloroform, C. P., and 5 ml. concentrated sulfuric acid. The chloroform and anhydride may be measured in a graduated cylinder and the sulfuric acid measured in an ordinary pipette. The reagent is kept at 16° C. and is usually prepared fresh for each set of ten samples. After the addition of the reagent to the sample, the solution was mixed thoroughly and transferred to the colorimeter tubes. The transfer is not necessarily quantitative, since the color intensity of the sample is to be measured. The colorimeter tube was stoppered immediately and placed in a beaker containing water at 16° C., and covered with a heavy towel. The beaker was placed in the constant temperature refrigerator at 16° C. The color intensity of the solution was read in the Evelyn photoelectrometer after about fifty minutes standing, using the 6200 Å light filter. The readings were checked at intervals of five minutes until a constant maximum reading was obtained. The blank reading was checked at 100 between each sample reading.

The standardization curve obtained by the above method on both pure cholesterol and cholesterol digitonide gave a value for K of $0.81 \pm 0.01$ for the former, and $0.82 \pm 0.01$ for the latter. The variation is $\pm 1.5$ per cent.

SUMMARY

Procedures are described for the extraction of tissue lipids and for the saponification of these lipids. The lipids of liver and adrenal tissues are completely extracted by 3 : 1 alcohol-ether in five hours at room temperature. Glycerides, cholesterol esters and methyl esters were shown to be completely saponified by heating for thirty minutes with a solution of potassium hydroxide in alcohol-ether. An improved titrimetric method for the estimation of fatty acids is described. Amounts of fatty acids from 1–10 mg. can be determined with an accuracy of $\pm 2.0$ per cent. A modification of the Schoenheimer and Sperry method for determination of free and combined cholesterol has been devised, after critical study of the best conditions for precipitation of cholesterol digitonide and the development of color in the Liebermann-Burchard reaction. The modified method is adapted for use in the Evelyn Photoelectrometer, and may be used in other colorimeters of this type.

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# STUDIES ON FRESH-WATER BRYOZOA

## XV. Hyalinella punctata Growth Data

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### INTRODUCTION

The fresh-water Bryozoa constitute a small group of widely distributed species. They may reproduce by some of the following methods: (1) budding, (2) chitin encased germinable bodies called statoblasts and hibernacula and (3) free-swimming larvae. Statoblasts occur in most fresh-water Bryozoa while hibernacula occur in only a limited number of species. These well-encased bodies often tide the species over periods of adversity, drought or winter cold. Tampering with the life cycle can be done most easily by experimenting with the statoblasts and hibernacula, since these bodies are hardy, can be chilled, dried or otherwise handled with relatively little care.

The purpose of the present article is to furnish data on the viability of floating statoblasts, known as floatoblasts (Rogick, Study XIV), the rate of formation and degeneration of individuals and the development and growth processes of floatoblast-derived colonies of *Hyalinella punctata*. The data herein given consist of observations on specimens whose life cycle had been experimentally delayed or tampered with in some way.

Difficulties of various types are encountered in studying living bryozoa. One is the matter of identification, which is particularly troublesome in the Plumatellidae of which *Hyalinella* is a member. Another difficulty is the feeding problem. A third is the maintenance of the colonies in the laboratory under such conditions as make it easy to study them conveniently under the microscope without disturbing them too much. This requires that they be grown in shallow dishes like Syracuse watch glasses or Petri dishes which can be placed readily under the compound microscope for examination. Colonies or coenoecia may or may not become attached to the substratum under laboratory conditions. Some attach within two or three days after germination of the floatoblasts which produced them, while others remain suspended from the surface film for a number of days. Eventually however, all will attach. Those which attach to the bottom of the dish are in a very favorable position for microscopic study. Those which attach to the sides or rim of the dish or to the surface film generally orient downward, necessitating their dislodgment with a dissecting needle so that they will lodge in a more favorable situation.

## DESCRIPTION OF FLOATOBLAST GROUPS

Study VIII (Rogick, 1939) reported a part of the *Hyalinella punctata* life cycle which dealt with the metamorphosis of the free-swimming larvae into young colonies. No larvae were produced by the colonies of the present study which deals with colonies derived from the following seven groups of *Hyalinella punctata* floatoblasts:

GROUP P. This group consisted of 42 floatoblasts dried a few days after collection and stored dry at room temperature for many months until the start of the experiment. None germinated.

GROUP Q. This consisted of 20 floatoblasts not dried until a number of months after collection, then stored dry, at room temperature, for a number of months until the start of the experiment. Half of them gave indications of germinating but did not complete the germination process to the point where visible polypides were protruded.

GROUP R. This consisted of floatoblasts chilled, then dried soon after collection and stored under these conditions in a refrigerator for a number of months until the start of the experiment. Of the 135 floatoblasts set out 96 germinated.

GROUP S. This consisted of floatoblasts remaining in water at room temperature for a number of months, then stored wet, i. e., in water in a refrigerator for several months until the start of the experiment. Of the 42 set out 12 germinated.

GROUP T. This consisted of 25 floatoblasts which remained in water at room temperature for a number of months until the start of the experiment. Five or one-fifth germinated.

GROUP U. This consisted of floatoblasts remaining in water for many months at room temperature after which they were dried and stored dry in a refrigerator until the start of the experiment. One out of 45 germinated.

GROUP V. This consisted of 14 floatoblasts which were dried sooner after collection than were those of Group R and which were stored dry in a refrigerator for a number of months before the start of the experiment. None germinated.

Briefly, Groups P, Q and T were not subjected to chilling and storage in the refrigerator at any time during the experiment. Groups R, S and V were. Group U was subjected to chilling and storage at low temperatures for only a part of its dormant period. Also, Groups P, R and V were stored in the dry state during their dormant period while Groups S and T remained wet throughout their entire period. Groups Q and U were subjected to drying only after the statoblasts had remained free in water for a number of months.

The official "start" of each experiment was the removal of floatoblasts from their places of storage and their immersion in tap water at room temperature for purposes of germination.

Three sets of floatoblasts, P-1, P-2 and P-3, were used in the P Group. Sets P-1 and P-3 were collected from Westtown Pond in Westtown, Chester County, Pennsylvania, on Sept. 9, 1940, dried four days thereafter and stored dry at room temperature in corked vials on a closet shelf until wanted for experimentation. Set P-2, collected from Beechmont Lake in New Rochelle, New York, on Aug. 8, 1937, was similarly treated but was dried eight days instead of four after collection. The method of desiccation was the same as described in Studies VII and XI. Sets P-1 and P-2 were "started" or immersed in tap water on June 29, 1941, and Set P-3 on Feb. 3, 1943, so that germinations might take place. Since none did and none seemed likely to, Sets P-1 and P-2 were discontinued 33 days after the start of the experiment and Set P-3 was discontinued 116 days after the start or immersion. Table I gives additional data on the conditions under which each set of this and other groups was kept.

Like floatoblasts of Sets P-1 and P-3 those of Groups Q, R, S, T, U and V were collected at Westtown Pond on Sept. 9, 1940.

At the time of collection a number of floatoblast-filled colonies of *Hyalinella punctata* were brought indoors from the pond. These colonies in due time normally released a large number of floatoblasts which were placed in a pint Mason jar with some *Elodea* and water. This jar was kept indoors at a comfortable room temperature from the time of collection until Aug. 2, 1941, or for a period of 327 days or 10.8 months, throughout the autumn, winter, spring and part of the summer. From this jar came the floatoblasts which were used in Groups Q, S, T and U.

Those which were to be used in the Q Group were removed from the jar on Aug. 2, 1941, dried, then stored dry in a corked vial on a closet shelf at room temperature for 18 months, until Feb. 3, 1943. They were then "started" or immersed in tap water and kept at a comfortable room temperature throughout their germination period. Half of the statoblasts did give some indication of germinating. They reached the point where the two floatoblast valves separated sufficiently to permit a glimpse of a small ball of tissue between them. This tissue however did not grow beyond the borders of the valves nor did it develop any visible or protrusible polypides. This group was discontinued 47 days after immersion because the ones which had begun to germinate were dead for some time and the others gave no indication of germination.

Floatoblasts for Group S were removed from the same pint jar containing *Elodea* and water as were those of Group Q, on Aug. 2, 1941. Thus they had remained in liquid at a comfortable room temperature for 327 days or 10.8 months. They were then placed in a refrigerator where they remained in liquid at the bottom of a small corked bottle from that date till Jan. 24, 1943, an interval of 540 days or 17.7 months. They were removed from the refrigerator on that date 28.5 months after collection, immersed in a watch glass containing tap water and kept thus at a comfortable room temperature from then on. The temperature and other data for this group can be found in Tables I and II. Twelve of the 42 floatoblasts used in this group germinated very speedily, eleven of them within one day after immersion and the twelfth on the second day after immersion. Most of the colonies derived from these germinated floatoblasts were discontinued between 16 and 32 days after floatoblast immersion because degeneration, due to improper (green algal) and insufficient food, had set in and the colonies were too degenerate to be saved. One colony was accidentally crushed 31 days after floatoblast immersion. The remaining floatoblasts were discarded 78 days after immersion because they had failed to germinate. Please refer to Table I for additional data on the group as a whole and to Tables II and III for data on colonies from dishes numbered S-6a, S-6b, S-6c and S-6d. Each of these dishes contained one or more colonies, thus accounting for some of the extra numbers found in the various columns in Tables II and III.

Floatoblasts for Group T were removed from the same pint jar containing *Elodea* and water as were those of the preceding groups, where they had remained in liquid for 10.8 months at a comfortable room temperature. They were transferred from that container on Aug. 2, 1941, to a Syracuse watch glass containing tap water. This was the "start" of the experiment. Hence, they were not chilled or dried but remained in liquid at room temperature for the interval between collection and the beginning of the experiment. Five out of 25 floatoblasts germinated. The colonies although in good condition were discarded after nine days because circumstances beyond control made it impossible to care for and daily observe the colonies at that particular time, so the colonies had to be discontinued.

Floatoblasts for Group U were removed from the same pint jar as were those of preceding groups on Aug. 2, 1941, after they had remained in liquid for 10.8 months at room temperature. They then were dried and stored dry in a refrig-

## TABLE I

### Data on *Hyalinella punctata* Floatoblasts Used in This Study

| | Group P | | | Group Q | Group R Floatoblasts | | | | | Group S | Group T | Group U | | Group V |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Set P-1 | Set P-3 | Set P-2 | | Set R-1 | Set R-2 | Set R-3 | Set R-4 | Set R-5 | | | Set U-1 | Set U-2 | |
| A. What was done to the floatoblasts before immersion in tap water | Dried 4 days after collection and stored dry at room temperature | | Dried 8 days after collection | Dried after long interval of freedom | Chilled then dried soon after collection. Stored in refrigerator | | | | | Free for long interval, then chilled and stored in water in refrigerator | Always in water. Stored at room temperature | Free for long interval, then dried, chilled and stored dry in refrigerator | | Dried 4 days after collection and stored in refrigerator |
| B. No. of days floatoblasts were chilled | 0 | 0 | 0 | 0 | 244 | 263 | 289 | 863 | 632 | 540 | 0 | 306 | 540 | 840 |
| C. Temperature ranges at which floatoblasts were kept in refrigerator | — | — | — | — | 7-11.2° C. | 7-11.2° C. | 7-11.2° C. | 7-13.5° C. | 7-11.2° C. | 7-13.5° C. | — | 7-13.5° C. | 7-13.5° C. | 7-13.5° C. |
| D. No. of days floatoblasts were kept in dry state | 289 | 873 | 1412 | 550 | 227 | 246 | 272 | 846 | 615 | 0 | 0 | 306 | 540 | 840 |
| E. Dates during which floatoblasts remained dry | IX-13-1940 to VI-29-1941 | IX-13-1940 to II-3-1943 | VIII-16-1937 to VI-29-1941 | VIII-2-1941 to II-3-1943 | IX-30-1940 to V-15-1941 | IX-30-1940 to VI-3-1941 | IX-30-1940 to VI-29-1941 | IX-30-1940 to 1-24-1943 | IX-30-1940 to VI-7-1942 | — | — | VIII-2-1941 to VI-4-1942 | VIII-2-1941 to I-24-1943 | IX-13-1940 to I-1-1943 |
| F. Dates during which floatoblasts were in refrigerator | — | — | — | — | IX-13-1940 to V-15-1941 | IX-13-1940 to VI-3-1941 | IX-13-1940 to VI-29-1941 | IX-13-1940 to I-24-1943 | IX-13-1940 to VI-7-1942 | VIII-2-1941 to I-24-1943 | — | VIII-2-1941 to VI-4-1942 | VIII-2-1941 to I-24-1943 | IX-13-1940 to I-1-1943 |
| G. No. of days from collection till immersion in tap water for start of experiment | 293 | 877 | 1420 | 877 | 248 | 267 | 293 | 867 | 636 | 867 | 327 | 633 | 867 | 844 |
| H. No. of days from immersion to germination | — | — | — | 25 | 2—4 | 2—6 | 2—7 | 4—23 | — | 1—2 | 2 | 6 | — | — |
| I. No. of floatoblasts used | 10 | 20 | 12 | 20 | 37 | 42 | 7 | 36 | 13 | 42 | 25 | 10 | 35 | 14 |
| J. No. of floatoblasts which germinated | 0 | 0 | 0 | 10 (?)* | 32 | 35 | 5 | 24 | 0 | 12 | 5 | 1 | 0 | 0 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| K. No. of floatoblasts damaged or imperfect | 0 | 0 | 0 | 0 | 1 | 4 | 2 | 2 | 0 | 1 | 0 | 0 | 0 | 0 |
| L. Temperature ranges at which floatoblasts and their resulting colonies were kept after immersion | 24-30.5° C. (average, 27.1° C.) | 20.5-31° C. (average, 25.5° C.) | 24-30.5° C. (average, 27.1° C.) | 20.5-31° C. (average, 25.5° C.) | 20.5-30.5°C. | 20.5-30.5°C. | 20.5-30.5°C. | 20.5-30.5°C. | 20.5-30.5°C. | 20.5-31°C. (average, 25.5° C.) | 26-28° C. | 23.5-26.3°C. | 20.5-30.5°C. | 23-29.8°C. (average, 26.4° C.) |
| M. No. of days after germination that first polypides evaginated | — | — | — | — | 1—2 | 1—3 | 2 | 1—4 | — | 2 | 1—2 | 8 | — | — |
| N. No. of first polypides evaginated in this group or set | 0 | 0 | 0 | 0 | 32 | 34 | 5 | 23 | 0 | 12 | 4 | 1 | 0 | 0 |
| O. Total no. of polypides drived from each floatoblast-produced colony before its death or discard | 0 | 0 | 0 | 0 | 1 | 1—2 | 2 | 1—500+ See Tables II, III, IV | 0 | 1—18 | 1 | 1 | 0 | 0 |
| P. No. of days colony or germinated material remained alive after germination | — | — | — | 13 (?)* | 7† | 9—22 | 3—27† | 8—176 | — | 15—31 | 7 | 20† | — | — |
| Q. No. of days between germination and onset of ancestrula's degeneration | — | — | — | — | 6—7 | 6—12 | 3—10 | 5—21 | — | 12—21 | — | — | — | — |
| R. Fate of colonies | discontinued | discontinued | discontinued | discontinued | discontinued some alive, some degenerating at the time | degenerated | some died, some killed, some discontinued | degenerated | discontinued | one accidentally killed. Rest discontinued | discontinued while alive, before degeneration set in | discontinued while alive, before degeneration set in | discontinued | discontinued |

* The 10(?) germinations of Group Q are not exactly normal because although the valves did crack apart and a slight amount of germinative material did appear between them this material did not grow beyond the edge of the valves and produced no visible polypides.

0 Means none or not at all.

— Means there was no data on this, principally because no germinations took place but occasionally for some other reason.

† The † sign after numbers in horizontal line "P" means that some of the polypides could have lived longer if they had not been discontinued for some reason or other.

## TABLE II

### Rate of Polypide Evagination and Ancestrula Degeneration in Colonies of *Hyalinella punctata* Derived from Germination of $FG_1$

| | Colonies in Sets | | Dishes Containing Colonies of Set R-4 from Floatoblast Group R | | | | | | | | Colonies from Group S Floatoblasts | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R-2 | R-3 | R-4a | R-4b | R-4c | R-4d | R-4e | R-4g | R-4h & k | R-4l | S-6a & d | S-6b | S-6c |
| 1. No. days between statoblast immersion and: | | | | | | | | | | | | | |
| a. germination | 2 to 6 days | 2 to 7 days | 4 days | 5 days | 7 days | 8 days | 9 days | 11 days | 15, 18 days | 23 days | 1 day | 2 days | 1 day |
| b. evagination of 1st polypide | 4 to 7 | 4 to 9 | 5 | 7, 8* | 9, 11 | 9, 12 | 10 to 13 | 12 | 20 | 25 | 3 | 4 | 3 |
| c. evagination of 2nd polypide | 10 to 19 | 18 | 14 | 13, 14* | | 18 to 23 | 16, 17 | 18 | 26 | 26 | 16 | 21 | 19 to 21 |
| d. evagination of 3rd polypide | | | 22 | 15 | | 21 to 26 | 22, 23 | 21 | | 28 | | 28 | 21 to 23 |
| e. evagination of 4th polypide | | | 23 | 16 | | 25 | 23 | 25 | | | | | 23 |
| f. evagination of 5th polypide | | | 23† | 17† | | 26† | 24† | 25 | | | | | 24 to 26† |
| 2. No. of days between germination and degeneration of ancestrula | | | 21 | 15 | 5, 9 | 16 to 21 | 11 to 18 | 13 | 10 | 8 | 12, 13 | 19 | 20, 21 |
| 3. No. of days between evagination of 1st and 2nd polypides | 6 to 12 | 9, 10 | 9 | 6 | | 9 to 14 | 4 to 6 | 6 | 6 | 1 | 13 | 17 | 16 to 18 |
| 4. No. of days between evagination of 2nd and 3rd polypides | | | 8 | 1, 2 | | 3 | 5, 6 | 3 | | 2 | | 7 | 2, 3 |
| 5. No. of days between evagination of 3rd and 4th polypide | | | 1 | 1 | | 1 | 1 | 4 | | | | | ½ to 2 |

* One of the two colonies in Dish No. R-4b was lost 16 days after floatoblast immersion. Thenceforth the remaining long lived one was referred to as Colony R-4b.

† Further evagination data on these colonies are shown in Table III.

Note: Dishes R-2, R-3, R-4b, R-4c, R-4d, R-4e, R-4g, S-6a and S-6c all contained more than one colony at the start, thus accounting for some of the ranges of figures in some of spaces.

erator until needed for experimentation. One June 4, 1942, 306 days later, ten statoblasts, constituting Set U-1, were removed from the refrigerator, immersed in a watch glass containing water and kept at a comfortable room temperature. One of the ten germinated. The resulting colony was discontinued 26 days after immersion while still alive although beginning to degenerate due to improper and insufficient food. The non-germinated floatoblasts likewise were discontinued at the same time. On Jan. 24, 1943, 867 days after collection or after 540 days of desiccation and chilling, 35 floatoblasts, constituting Set U-2, were removed from the refrigerator, immersed in a watch glass containing tap water and kept at room temperature. None of this U-2 Set germinated even though it was kept under observation for 126 days from that time.

TABLE III

RATE OF POLYPIDE EVAGINATION IN *Hyalinella punctata*
(Continued from Table II)

| Number of Days Between Floatoblast Immersion and Evagination of the | Colony R-4a | Colony R-4b | Colony R-4d | Colony R-4e | Colony S-6c |
|---|---|---|---|---|---|
| | *days* | *days* | *days* | *days* | *days* |
| 6th polypide | 25 | 18 | 26, 27 | 24 | 24, 26 |
| 7th polypide | 26 | 18 | 27 | 26 | 25 |
| 8th polypide | 26 | 18 | 27 | 26 | 26 |
| 9th polypide | 27 | 18 | 27, 28 | 26 | 26 |
| 10th polypide | 28 | 20 | 28 | 27 | 26 |
| 11th polypide | 28 | 21 | 29 | 27 | 26 |
| 12th polypide | 31 | 21 | 29 | 27 | 27 |
| 13th polypide | | 21 | 29 | 27 | 27 |
| 14th polypide | | 21 | 29 | 29 | 27 |
| 15th polypide | | 21 | 29 | 32 | 27 |
| 16th polypide | | 21 | 29 | 32 | 28 |
| 17th polypide | | 21 | | 35 | 28 |
| 18th polypide | | 22 | | 35 | 29 |
| 19th polypide | | 22 | | 35 | |
| 20th and 21st | | 22 | | | |
| 22nd to 26th | | 23 | | | |
| 27th to 31st | | 24 | | | |
| 32nd to 45th | | 25* | | | |

NOTE: Data on Colony R-4b is continued in Table IV.

* This 25 days after statoblast immersion is the same as 20 days after statoblast germination, since there was a 5 days' difference between these two conditions.

Group V consisted of fourteen floatoblasts which were dried four days after collection, then placed in a refrigerator where they remained dry for 840 days, until Jan. 1, 1943, when they were taken out, immersed in tap water and kept at room temperature for 67 days, but no germinations took place so the group was discontinued at the end of that interval. Additional data on this and on the preceding groups may be obtained from Table I.

Originally all the statoblasts of a particular group or set were placed in a watch glass which was labelled with the capital letter of the group and the number of the set, thus: R-4. As soon as any floatoblasts gave the faintest indication of

approaching germination they were removed to another watch glass which would be distinguished from the first by an additional letter after the number, thus: R-4a. When additional floatoblasts of dish R-4 began to germinate another watch glass labelled R-4b would be prepared for the newly germinated statoblasts, and so on. Generally, when several statoblasts germinated on the same day they would all be placed in the same watch glass unless their numbers were so great as to make that confusing or impractical. Such a procedure explains the numerous figures given in column R-4d of Table II, because watch glass R-4d contained a total of nine colonies, some of which had different polypide evagination rates.

Group R furnished some of the most interesting results for the present study because out of its 135 floatoblasts, which were placed in five Sets, R-1, R-2, R-3, R-4 and R-5, 96 germinated and some of them produced long-lived colonies. The R Group consisted of floatoblasts which were placed in a loosely capped vial containing some water in a refrigerator four days after collection. The vial accidentally tipped over, leaving the floatoblasts dry by Sept. 30, 1940, or 21 days after collection. The floatoblasts remained dry in the refrigerator until the beginning of the experiment (please refer to Table I).

Although colonies which developed from the R floatoblasts were very promising material, quite a number of them degenerated too soon because of the food

TABLE IV

FURTHER DATA ON *Hyalinella punctata* COLONY R-4b. THE $FG_1$ STATOBLAST WHICH PRODUCED THIS COLONY GERMINATED 5 DAYS AFTER IMMERSION

| 1943 Date | No. of days from time of germination to date at left | No. of colony fragments | No. of polypides evaginated in any single fragment on date at left | | Total No. of polypides evaginated in all fragments on date at left | No. of mature floatoblasts released or torn out at given date |
|---|---|---|---|---|---|---|
| | | | Maximum | Minimum | | |
| II–18 early | 20 | colony intact | 44 | | | |
| II–18 later | 20 | 2 | 36 | 8 | 44 | |
| II–19 | 21 | 2 | 37 | 10 | 47 | |
| II–20 | 22 | 2 | 38+ | 14 | 52+ | |
| II–26 | 28 | 1 | 38+ | — | 38+ | 2 |
| II–28 | 30 | 1 | 38+ | — | 38+ | 1 |
| III– 3 | 33 | 1 | 63+ | — | 63+ | |
| III– 5 | 35 | 1 | 66+ | — | 66+ | |
| III–10 | 40 | 1 | 55+ | — | 55+ | |
| III–12 | 42 | 1 | Polypides temporarily withdrawn, so can not tell which are alive and which are degenerating. Some degeneration did take place during these intervals. | | | 5 |
| III–13 | 43 | 1 | | | | 5 |
| III–14 | 44 | 1 | | | | 8 |
| III–15 | 45 | 1 | | | | 4 |
| III–16 | 46 | 1 | | | | 13 |
| III–17 | 47 | 1 | | | | 1 |
| III–18 | 48 | 1 | | | | 3 |
| III–22 | 52 | 1 | | | | 1 |
| III–24 | 54 | 1 | 55 | | 55 | |
| III–29 | 59 | 1 | 50 | | 50 | |
| IV– 1 | 62 | 1 | 70 | | 70 | |
| IV– 2 | 63 | 1 | 76 | | 76 | |

TABLE IV—[*Continued*]

| 1943 Date | No. of days from time of germination to date at left | No. of colony fragments | No. of polypides evaginated in any single fragment on date at left | | Total No. of polypides evaginated in all fragments on date at left | No. of mature floatoblasts released or torn out at given date |
|---|---|---|---|---|---|---|
| | | | Maximum | Minimum | | |
| IV–4 | 65 | 1 | 84 | | 84 | |
| IV– 6 | 67 | 1 | 97 | | 97 | 1 torn out |
| IV– 8 | 69 | 1 | 99 | | 99 | 1 torn out |
| IV–12 | 73 | 2 | 78 | 13 | 91 | |
| IV–13 | 74 | 2 | 76 | 12 | 88 | |
| IV–17 | 78 | 3 | 57 | 14 | 109 | |
| IV–18 | 79 | 6 | 52 | 1 | 110 | |
| IV–19 | 80 | 7 | 45 | 1 | 137 | |
| IV–23 | 84 | 8 | 41 | 6 | 137 | 1 |
| IV–24 | 85 | 8 | 33 | 5 | 130 | 3 |
| IV–25 | 86 | 8 | 46 | 5 | 150 | |
| IV–26 | 87 | 8 | 51 | 5 | 159 | 1 |
| IV–29 | 90 | 8 | 67 | 7 | 189 | 1 |
| IV–30 | 91 | 8 | 76 | 11 | 204 | 2 |
| V– 1 | 92 | 9 | 92 | 11 | 253 | 1 |
| V– 2 | 93 | 9 | 99 | 14 | 288 | |
| V– 3 | 94 | 11 | 100 | 1 | 351 | |
| V– 5 | 96 | 13 | 122 | 1 | 395 | |
| V– 6 | 97 | 14 | 108 | 1 | 365 | |
| V– 7 | 98 | 15 | 120 | 1 | 400 | |
| V– 9 | 100 | 15 | 115 | 1 | 398 | 1 |
| V–13 | 104 | 10 | 159 | 6 | 471 | |
| V–17 | 108 | 11 | 144 | 6 | 491 | |
| V–19 | 110 | 14 | 107 | 4 | 434 | |
| V–22 | 113 | Progressive degeneration due to food and neglect | | | | 4 |
| V–30 | 121 | 11 | 113 | 1 | 282 | |
| VI– 3 | 125 | 12 | 87 | 1 | 177 | 1 |
| VI– 8 | 130 | 12 | 68 | 2 | 204 | |
| VI–14 | 136 | 14 | 55 | 2 | 194 | 1 |
| VI–17 | 139 | 14 | 38 | 2 | 170 | 2 |
| VI–19 | 141 | | | | | 8 |
| VI–21 | 143 | 13 | 28 | 1 | 136 | 6 |
| VI–25 | 147 | | | | | 3 |
| VI–27 | 149 | 20 | 11 | 1 | 67 | |
| VI–30 | 152 | 28 | 12 | 1 | 87 | |
| VII–16 | 168 | 14 | 3 | 1 | 21 | 11 |
| VII–18 | 170 | 9 | | | 16 | |
| VII–19 | 171 | 11 | | | 14 | |
| VII–24 | 176 | Colonies or fragments discontinued | | | | 1 |

problem. Set R-1 was fed green algae and other aquarium scrapings but failed to thrive on this diet. Degeneration set in early and the R-1 colonies were discontinued nine days after statoblast immersion. Set R-2 received a similar diet with the same results. Because the R-2 colonies were degenerating they were discarded 26 days after floatoblast immersion. Set R-3 colonies also did not thrive well, some of them degenerating because of green food, others because of lack of food and presence of enemies. Some specimens were discontinued as early as seven days after floatoblast immersion and others as late as 33 days. The colonies were discontinued when still alive when it became evident that degeneration had progressed so far that it was useless to keep them longer. Set R-4 was an unusually productive one, giving rise to a good number of polypides and to some long-lived colonies. Except for one lot which was accidentally destroyed sixteen days after statoblast immersion the colonies lasted a number of days. Some were discarded after a well advanced stage of degeneration on the following days after statoblast immersion: 28, 31, 32, 35 and 51 days. Some colonies fragmented, i. e., polypide groups would become separated from the main body of the colony so that in time there would be a number of living fragments rather than one main colony (refer to Table IV). The longest lived fragments died from lack of adequate care in the final few weeks and from degeneration 181 days after statoblast immersion. This length of time is a record under laboratory conditions. Formerly, in Study III, two *Lophopodella carteri* colonies were kept alive 161 and 163 days after germination while in the present Study the most hardy *Hyalinella punctata* colony and its fragments (R-4b) lived 176 days after germination. Both species could probably have lived somewhat longer if it had not been necessary to move them to other localities or if they could have been cared for properly during and after removal. Data on the growth and progress of the long-lived colony R-4b can be found in Tables I, II, III and IV. Set R-5 was discontinued 23 days after the start of the experiment although no germinations took place in it. Table I gives some data on this last Set.

## DESCRIPTION OF REARING METHODS

Experimentation on delayed development, on the effects of desiccation, chilling or other factors can best be carried out on statoblasts since these bodies are far hardier and firmer than any other parts of the bryozoan. Statoblasts form and mature within the colony, then may be released soon thereafter or else may be retained within the zoarium after the death and degeneration of the polypides which produced them. Some writers maintain that a rest period is necessary before statoblasts germinate; others doubt it. Statoblasts to be used for the experiment may be dissected directly out of living colonies or may be collected after the colonies had normally released them by expulsion or by division of the colony. The advantage of dissecting them out of living colonies is that one can be sure of their source, particularly if several closely related species are in the same collection. The advantage of allowing colonies to normally shed their statoblasts in the laboratory is that the colony stocks will not be prematurely destroyed, the statoblasts are more likely to be fully mature and finally, much less time is required to collect only shed statoblasts than to dissect them out carefully. All *Hyalinella* statoblasts used in the present study were collected after being normally shed by their colonies. Those used in Set P-2 were collected in Beechmont Lake, New Rochelle, New York, on Aug. 8, 1937. Those used in all the other sets and groups were collected in Westtown Pond, Westtown, Pa., on Sept. 9, 1940. For a more complete description of the collecting sites please refer to Studies IX and XIV.

Due to their smaller size floatoblasts of *Hyalinella* and *Plumatella* are less favored for experimentation than those of *Cristatella*, *Pectinatella*, *Lophopus* or *Lophopodella* because they are harder to keep track of or handle.

When an experiment was begun, the dried floatoblasts of a particular group or set were removed from the vials or containers in which they had been stored to Syracuse watch glasses or Petri dishes containing tap water which was later replaced by culture, pond or aquarium water as soon as the statoblasts gave any indication of germinating. This was known as "immersion." However, in the P and V Groups, since no floatoblasts germinated, tap water alone was used. In the other groups tap water was soon replaced by more suitable liquid and food because many statoblasts showed signs of approaching germination.

The specimens were observed once or twice daily with a compound microscope. As a rule dried floatoblasts upon immersion for germination are likely to remain on top of the surface film. When possible they were pushed down with a dissecting needle until they were beneath the surface film. Most of them floated up to the surface film immediately thereafter and remained around the rim of the glass just beneath the film even a number of days after germination.

When the floatoblasts seemed to be near the point of germination tap water was replaced by other water containing food material. The following different foods were tried in some of the sets or groups: green algae (desmids, etc.) from aquarium scrapings, organic debris from the bottom of various culture dishes, tiny ciliates and flagellates, *Paramecia*, small quantities of fresh yeast cake and bacterial scum from culture waters which had one of the following food substances added to them: lettuce, yellow cornmeal, oatmeal, bread crumbs, rice and other cereals. The germinated colonies rejected *Paramecia* but not the very small flagellates and ciliates. The cornmeal and yeast cultures proved the most satisfactory because the colonies seemed to thrive best when these were used, particularly so when small lumps of yeast were placed directly in the dishes containing the colonies. The colonies were maintained on a varied diet rather than on one substance alone because it was found to produce healthier colonies. When colonies began to slow down in polypide production or began to show signs of degeneration the diet was changed because it was more important at the moment to keep the colonies alive than to test the efficacy of the various food products. Additions of small quantities, fragments, of fresh yeast seemed to keep the colonies flourishing. The use of the green algae alone had the opposite effect. It caused the colonies either to degenerate after a short time or to slow down their polypide multiplication. The slowing down was evident in statoblast development. In some colonies floatoblasts were formed and of fair size and degree of development then failed to carry the developmental process through to completion either because of the degeneration of polypides or for other reasons. Among the most obvious characteristics of degeneration was the stunting effect. The tentacles began to shorten and thicken. The pattern of ciliary action was disrupted. The entire zooid shortened and became smaller. In time the lophophore withdrew permanently. The polypide shortened, thickened and condensed to a brownish-orange colored ball known in literature as the "brown body." In some colonies all the polypides degenerated until there was nothing left but a small bag of coenoecial wall which was progressively reduced in size until it was hard to find among the debris of the dish.

Methods of staving off degeneration were (1) to change the diet if necessary, (2) to keep the colony and the dish in which it was growing as clean as conditions would permit and (3) to aerate the water in the colony dish with a pipette when necessary. Colonies were kept clean of old fecal pellets, debris, scum and other ectocyst-covering accumulations by removing this material with a pipette or by scraping with a dissecting needle or fine forceps. Sometimes this accumulation was so great that it covered the entire colony and had large holes here and there through which the introvert could be protruded, (fig. 1). The accumulation was removed daily when possible. The scraping was done very gently to prevent crushing, puncturing or damaging the colony. Occasionally a colony was constricted or divided by this scummy covering. A colony was sometimes accidentally pulled

1
2
3
4
W
0
1
2
3 mm.
X
0
0.2mm.

apart at this thinned or constricted area without any apparent ill effect on subsequent growth. When that was done, the ends of the broken connection closed up and healed very speedily, provided that the connection was narrow and thin at the time of division. Not only was it the colonies which needed cleaning but also the inner surfaces of the watch glasses and Petri dishes because the debris, stale food, fecal pellets, etc., accumulated at the bottom, generally in the vicinity of the colony. This cleaning was done either by use of a pipette or by emptying most of the water from the dish, hurriedly wiping the inner surface with a clean cloth, taking care not to injure or dislodge the attached colony. Then fresh culture water from a small aquarium and a few tiny lumps of fresh yeast or drops of food culture were added to the dish which contained the bryozoan colony.

## OBSERVATIONS

### *Germination*

Within a few days, 1 to 25, after immersion of the various sets or groups of floatoblasts germinations took place in the usual manner. The statoblast valves separated slightly. Between them appeared a bag of tissue containing germinative material and yolk masses. A day or so later, (refer to Tables I and II), the germinated zooid was sufficiently developed to evaginate or protrude a tentacle-bearing lophophore. When evagination had taken place, the distribution of yolk particles was more discernible, (see figs. 5–7). Yolk occurred in extensive masses in the walls of the digestive tract, coenoecium and lophophore. It furnished food for the early developmental stages of the colony. In several instances it had disappeared by the third day after germination while in a few colonies it lasted till the fourth, fifth and even sixth day after germination of the *Hyalinella punctata* statoblasts. In *Lophopodella carteri* the yolk was visible from four to seven days after statoblast germination. Brooks (1929) working with *Pectinatella magnifica* found that the yolk mass had almost disappeared from the *P. magnifica* colonies by eight days (p. 431) and that some young colonies could live for two weeks on the yolk stored in their bodies (p. 435).

### *Ancestrula*

The first zooid to evaginate is known as the ancestrula. In a normal *H. punctata* colony, the ancestrula had a smaller number of tentacles than did successive zooids. Marcus (1941, p. 150) observed a similar condition in *Stolella evelinae* ancestrulae. Therefore, a count of the tentacle number was one method of checking whether one was dealing with the ancestrula or with one of its successive

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## EXPLANATION OF FIGURES IN PLATE I

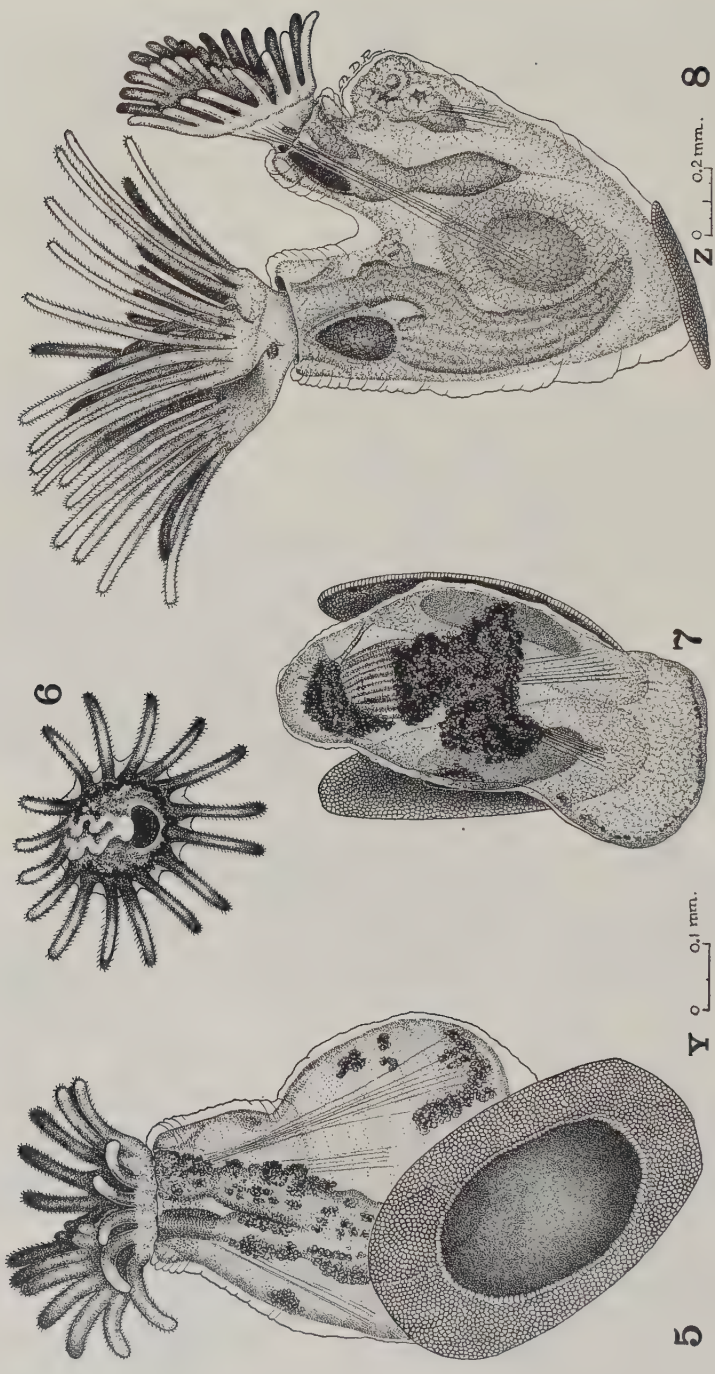
The species depicted in all the figures is *Hyalinella punctata*. All except Figures 2 and 4 were drawn with the aid of a camera lucida.

Fig. 1. Colony R-4b as seen from above on IV-6-1943, or 67 days after germination. The central part of the colony is quite opaque and heavily covered with debris and accumulations so that the zooids in it are hard to see or to count. The transparent ectocyst is best seen around the long branches. The entire colony is attached to the substratum at this time. Drawn to Scale W.

Fig. 2. A diagram of the "twin" polypides as they appeared in Colony R-4b on the 65th day after floatoblast immersion or on III–30–1943. View is of anal side. These polypides were already several days old at this time. Both polypides are using the same invaginated fold and their outer retractors are evident. Not drawn to any scale.

Fig. 3. An immature sperm mass on the funiculus of Colony R-4b's second polypide as seen on II–8–1943 or on the 10th day after germination. Drawn to Scale X.

Fig. 4. Three statoblasts developing on the funiculus of a Colony R-4b polypide. The oldest statoblast is proximal to the tip of the digestive tract. Drawn to Scale X on II–14–1943.


5
6
7
8
X
0,1 mm.
Z
0,2 mm.

buds. The average tentacle number in ancestrulae of 34 *H. punctata* colonies was approximately 28, the maximum number 34 and the minimum 16+. The 16+-tentacled individual had evaginated somewhat prematurely. It had a very small lophophore and short, still poorly differentiated tentacles. The average tentacle number in seventeen normal successive zooids was 44, the maximum 50 and the minimum 32. This last figure was for a very immature polypide which in less than two days increased its number to 44 tentacles. Many more examples than seventeen were observed and counted but because the results were in complete agreement with the figures given above they were not formally recorded. In general it can be said that the average number of tentacles remained smaller in the ancestrula throughout its life span than in the successive polypides although it did increase slightly in both as they matured. For example, one ancestrula which had nineteen tentacles upon evagination added two more within ten hours. Another ancestrula added eight more tentacles to its 24 within four days. Some of the successive polypides likewise added a few tentacles after evagination. In one set of experiments, Colony R-4e, where the food supply was very unsatisfactory, (green food), the colonies and successive polypides were very stunted and the tentacle number was unusually small—sixteen to twenty tentacles. This was an abnormal condition.

The ancestrulae of this study and of Study VIII (p. 208) were the first polypides to degenerate, under normal conditions. The ancestrulae degenerated from 13 to 31 days (average 24.2 days) after statoblast immersion in the case of sixteen colonies and from 8 to 21 days (average 14.3 days) after germination. Some ancestrulae degenerated without giving rise to successive polypides while others survived until the colony had seventeen individuals before degenerating. Those which degenerated early were those which were fed an inadequate or unsuitable food (green algal material). This not only destroyed the ancestrula but the colony as well, sooner than was expected.

The degenerated ancestrulae shrank in size, withdrew into the coenoecium, darkened and either broke up inside or became pinched off. At any rate they

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## EXPLANATION OF FIGURES IN PLATE II

Fig. 5. Side view of an S-6a ancestrula as seen on I–27–1943, two days after floatoblast germination or three days after immersion. Large irregular masses of yolk are evident in the wall of the digestive tract, around the base of the long retractor strands and in the endocyst. The ancestrula had only twenty tentacles at this particular time but later increased the number. Only one statoblast valve appears in the diagram. The other valve, not shown here, is directly opposite and hidden by the one pictured. Drawn to Scale Y.

Fig. 6. The tentacular crown of the ancestrula of Fig. 5 as seen from above. The mouth is overhung by a rather small epistome, shown here as a white flap, which enlarges later on. A mass of yolk, the irregular black ring of the diagram, is seen around the bases of the tentacles at this early stage but later disappears. It is also present around the epistome but could not be shown here conveniently. The tentacles number 20, increased to 24 one day later (I–28–1943) and to 28 by the day following. Drawn to Scale Y.

Fig. 7. A small retracted R-4e Colony drawn one day after germination or ten days after immersion. A very small amount of yolk is seen dispersed in the basal endocyst region. A large yolk mass, (dark central irregular mass in the center of the diagram), appears around the digestive tract while a smaller amount is seen directly above, in the invaginated fold region. Statoblast valves appear on either side of the germinated colony. Drawn to Scale Y.

Fig. 8. Side view of Colony R-4b. Drawn on II–6–1943 or eight days after germination or thirteen days after immersion. The larger of the two evaginated polypides is the ancestrula. It has ±32 tentacles but not all can be seen from this view. The second or smaller polypide has 40 tentacles differentiated at this time. The two statoblast valves are shown, one in full-face view at center back and the other in partial edge view at the base of the colony. The retractors and funiculus of the ancestrula have been omitted. Drawn to Scale Z.

disappeared within a few days. Polypides other than the ancestrulae degenerated in the same manner. The rate of degeneration and the time factor in their case however has not been studied.

*Successive Polypides*

Buds formed and evaginated at a rapid rate when the food was adequate and suitable. To estimate the rate of bud formation it would have been more desirable to record the initial appearance of each bud germ or rudiment but from the practical point of view it proved less satisfactory and less easy than the method adopted, namely: noting the time when the bud had developed sufficiently to evaginate and take external food. In large colonies or in those covered with debris and various accumulations it was very hard to see buds forming and often those which were present might be mistaken for other things in a crowded colony. Bud formation and development in *Hyalinella* was very similar to the bud formation of other Plumatellidae. Buds at the time of evagination were well developed although small and short. Their lophophores were rather immature in that not all the tentacles had differentiated sufficiently from each other and from the substance of the lophophore to be easily counted.

The time interval between evagination of the ancestrula and the second polypide is generally slightly longer than the intervals between other pairs of successive polypides. It ranged from one to eighteen days. The interval between evagination of the second and third polypides was from one to eight days, while between the third and fourth polypides it was from one-half to four days. The interval between evagination of the fourth and fifth zooids was from a few hours to three days while the interval between emergence of the fifth and sixth was from a few hours to one day. From the sixth polypide onward the intervals were shorter and shorter, as a glance at Tables II and III will show. The reason for this was that a number of buds were forming then maturing at approximately the same time.

The time interval between statoblast immersion and the evagination of the second polypides in colonies of Table II was from 10 to 26 days or averaged 17.7 days for 31 colonies. The interval between statoblast immersion and evagination of the third polypides was from 15 to 28 days. For the fourth polypides it was from 16 to 25 days; for the fifth polypides 17 to 26 days; for the sixth, seventh and eighth it ranged from 18 to 27 days; for the ninth it was from 18 to 28 days; for the tenth it was from 20 to 28; for the eleventh, thirteenth and fourteenth it was from 21 to 29; for the twelfth it was from 21 to 31; for the fifteenth and sixteenth polypides it was from 21 to 32 and for the seventeenth from 21 to 35 days after statoblast immersion. Tables II and III show some of this data.

*R-4b Colony*

The time interval for evagination of *Hyalinella* polypides beyond the nineteenth polypide was based on data obtained from one of the colonies of Dish No. R-4b and is shown in Table III. This colony's twentieth and twenty-first polypides evaginated on the 22nd day after statoblast immersion. Its twenty-second to twenty-sixth polypides evaginated on the 23rd day, its twenty-seventh to thirty-first polypides evaginated on the 24th day, its thirty-second to forty-fifth polypides evaginated on the 25th day. By the evening of the 25th day, the dish containing the R-4b colony was accidentally dropped and the colony was torn into two separate pieces, one fragment with 36 polypides and the other with 8. The ancestrula which began to degenerate five days previously accounts for the forty-fifth polypide.

Up to this time, the only individual of this particular colony in dish R-4b to degenerate had been the ancestrula and observations of the inner contents of the colony had been easy. Now, after the accidental separation of the colony, counting of polypides and degenerate individuals became more difficult because the larger

fragment was no longer attached to the substratum but had become a ball-shaped mass from which polypides protruded in all directions. For that reason, for several days thereafter polypide counts of the larger fragment were only approximate. The polypide counts of the smaller fragment were easy and accurate because that part of the original colony fortunately had not been torn loose from the substratum at the time of the accident. That fragment evaginated two additional polypides the next day and four more the day following, making a total of fourteen nicely evaginated buds. The third day after the tearing of the original colony the two fragments were still evaginating additional buds but degeneration had set in in some of the polypides, four in the smaller fragment and several in the larger. By the seventh day after the tearing all polypides of the smaller fragment were dead while the larger fragment was getting along apparently fairly well and had between 28 and 38 polypides nicely evaginated. The number is uncertain because 28 could be definitely counted from one view and there were a number on the other side which could not be counted accurately but were estimated to be about 10. From this time on there was a steady increase in the number of evaginated polypides which could be counted but the colony fragment remained unattached until the 52nd day from the time of statoblast immersion or the 27th day after the tearing of the colony.

In Colony R-4b there was noted on the 60th day after germination a rather rare and unusual pair of well-developed, evaginated polypides, twins, sharing a joint invaginated fold but having a partially or slightly separated tentacular sheath and so far as could be ascertained, one set of retractors on each side, moving both polypides as a unit in and out of the coenoecial sac (fig. 2). The twins evaginated, invaginated or unfurled their tentacles simultaneously, moving or acting as a unit. When one was disturbed with a dissecting needle both retracted and after recovery both evaginated as one. They were crowded together rather closely, side by side, but seemed very vigorous. Their digestive tracts were separate and complete but so close together that one could not determine if there were any retractor muscles between them. The tentacle sheath was larger than normal and so was the diameter of the duplicature or invaginated fold. The twins were still functioning nicely five days later. After that they were lost sight of because of the large number (84) of polypides in the colony at that time and because degeneration set in in some parts of the colony, resulting in masses of debris here and there over the colony. It is very probable that the twin polypides degenerated prematurely. At any rate they could not be found after that. These polypides were well developed, mature and several days old when first observed. One of the reasons why they had passed unnoticed up to that time was because of the large number of polypides evaginated in the colony at that particular time.

It would have been ideal to have been able to study a colony from germination through evagination and degeneration of every formed zooid. That would be an almost impossible feat under even the most ideal conditions in view of the fact that colonies may consist of hundreds of crowded individuals and may sometimes be opaque enough in some regions to make observations unsatisfactory.

After the R-4b Colony was torn in two and its larger fragment curled up into a ball or bag of evaginated polypides, (this was between the 20th and 47th day after germination of the colony or between the 25th and 52nd day after immersion of the original floatoblasts), it was no longer possible to count with any great accuracy the exact number of polypides which degenerated and evaginated during that interval. All that could be done with reasonable accuracy was to count the number of polypides which had their tentacles evaginated at any one given moment and to record this number. This means that identical counts for successive days or any days may be variously interpreted. They might mean that no change had taken place in the colony or they might mean that several polypides had degenerated and an equal number of new buds had evaginated their lophophores at about the

same time so that the count would seem identical. Table IV shows the number of polypides which had their tentacles out at the time of counting and does not take into consideration the number of polypides which had degenerated. The greatest number of *H. punctata* zooids "out" or evaginated at any one time was 491 in the aggregation of R-4b colony fragments. The largest number of *Lophopodella carteri* zooids out at any one time was 45 (Rogick, Study III, p. 462).

The larger of the two original fragments of Colony R-4b continued its development until the 73rd day after germination when it had 91 polypides in the evaginated state and 15 withdrawn, degenerating polypides. It then fragmented into two pieces, one with 13 polypides out and the other with 78 polypides out. From here on fragmentation continued until eventually, on the 152nd day after germination, there developed from the original fragment 28 colony fragments, several of which contained only a single polypide each. Many of these fragments had been the result of too fast growth of zooecial branches in various directions away from the colony center, followed by degeneration of some of the intermediate polypides or areas between branches. Others of these fragments have been the result of accidental severing from the colony during the cleaning process which consisted of scraping away debris or pellet accumulations or degenerated material from the ectocyst.

The fragmentation phenomenon is not a new thing. It has been found in other bryozoan species: in *Stolella evelinae* by Dr. Marcus (1941, pp. 92, 150), in *Lophopodella carteri* by the writer (1935, p. 462) and very probably in other species by earlier workers. In general, the maximum number of fragments of Colony R-4b in existence at any one time was 28 and the maximum number of polypides evaginated in one fragment at a particular time was 159. For a more detailed account of the number of fragments and the maximum and minimum numbers of evaginated polypides in the fragments please refer to Table IV.

The fragmentation was most pronounced after the culture water had become too rich. Also at such times the amount of degeneration was quite considerable. In general the change from spoiling culture water to fresher media with the addition of a small amount of fresh yeast was the signal for accelerated polypide development and evagination. Examples are shown in Table IV; for example: fresh culture water and yeast were added to the colony fragments on the 97th day after germination and the very next day the number of evaginated polypides was 35 more than on the previous day. Another example of colonies speeding up polypide production and evagination was when fresh culture water and yeast were added to the colonies on the 101st day after statoblast germination, then every other day practically for about a week, until the number of evaginated polypides had risen from 395 on the 101st day to 491 polypides on the 108th day after statoblast germination, or the difference of 96 polypides in seven days. Sometimes, however, the addition of yeast did not arrest degeneration.

Too much or too little food, lack of care, neglect, rough handling, puncture or injury with dissecting needle and failure to keep culture water reasonably fresh contributed greatly to degeneration of polypides. See data from April 8 to 13 and from May 17 to July 19 in Table IV. After a colony began to show signs of regression, the dish was cleaned and fresh culture water and yeast were added to try to bring the colony back to normal. After such treatment there was some "revival" of the colony or an increase in the number of polypides, as data from May 9 to 17, from June 3 to 8 and from June 27 to 30 in Table IV show. The colonies were packed for transfer from New Rochelle, N. Y., to Woods Hole, Mass., on June 30 and received very little attention or care after transfer till the degeneration of the various fragments of the colony on July 24. Perhaps, if it had been possible to properly care for the colony fragments during the interval they might have survived for a considerably longer period.

### *Polypide Degeneration*

The polypide degeneration rate was greatly influenced by improper food, fouling of the culture water, accidental injury and the presence of enemies like rotifers and oligochaetes.

Table V gives the degeneration data for various groups, sets or colonies. It indicates when the first definite signs of degeneration appeared rather than the day when the polypides actually died. Sometimes there was a difference of one to four days between onset of degenerative processes and eventual death of a polypide, and a difference of three to seven days between onset of degeneration and complete disappearance of the polypide.

TABLE V

DEGENERATION RATE OF POLYPIDES

| Group or Dish or Colony Number | Polypide Number | Number of Readings, 1 per polypide | Number of days from immersion to degeneration of that particular polypide | | |
|---|---|---|---|---|---|
| | | | Maximum | Minimum | Average |
| R-1 | ancestrula | 28 | 10 | 9 | 9.1 |
| R-2 | ancestrula | 29 | 15 | 8 | 11.6 |
| R-3 | ancestrula | 5 | 17 | 5 | 9.8 |
| R-4 | ancestrula | 18 | 31 | 12 | 23.7 |
| S-6 | ancestrula | 12 | 22 | 12 | 15.4 |
| R-2 | second | 11 | 26 | 13 | 19.4 |
| R-3 | second | 2 | 30 | 30 | 30.0 |
| R-4 | second | 14 | 31 | 22 | 27.9 |
| S-6 | second | 5 | 29 | 23 | 26.0 |
| R-4 | third | 7 | 32 | 26 | 30.0 |
| S-6 | third | 2 | 29 | 27 | 28.0 |
| R-4e | fifteenth | 1 | 37 | 37 | 37.0 |
| R-4e | sixteenth | 1 | 43 | 43 | 43.0 |
| R-4e | seventeenth to nineteenth | 1 each | 49 | 49 | 49.0 |

In Sets R-1 and R-3, the food was unsuitable and caused the early degeneration of the first zooids and prevented the colonies from developing many polypides. The same was true for colonies in dishes R-4a, R-4c, R-4d and S-6. At times some factor or combination of factors would cause the simultaneous degeneration of several polypides in the colony.

Broadly speaking, the ancestrulae or first polypides degenerated from 5 to 31 days after floatoblast immersion or from 3 to 21 days after statoblast germination. The second polypides degenerated from 13 to 31 days, the third polypides from 26 to 32 days and the fourth polypides from 27 to 33 days after floatoblast immersion. It is quite possible that the maximum number of days given in Table V is shorter than it would have been had food and other growth conditions been better.

Rather unexpectedly, the longest lived colony, R-4b, furnished the least accurate data on polypide degeneration, largely because it was accidentally torn in two on the 25th day after floatoblast immersion and because of its subsequent fragmentations. Up to that time, the 25th day after floatoblast immersion, only one polypide, the ancestrula, had degenerated. Its degenerative processes had set in or begun five days previously. Thenceforth, it became difficult to tell

which polypide was the second, third, fourth, etc. Moreover, the larger fragment had developed an opaque surface, was unattached and in the shape of a ball for some time after that, so that accuracy in recording degeneration of successive polypides was impossible. Therefore no reliable record was obtained of degeneration of polypides from the 25th day after floatoblast immersion for this particular colony.

In some colonies the ancestrula was the only polypide to evaginate and degenerate. In other colonies a number of polypides, even as many as fourteen, were produced and evaginated before the ancestrula showed definite signs of degeneration.

### *New Terms*

The entire foregoing account of germinating statoblasts was based on a floatoblast generation which had originated or was produced outdoors, under natural conditions, before collection. For reasons which will soon be apparent it was necessary to distinguish the various generations of statoblasts which were produced by assigning to each generation a symbol or name so that it would be possible to refer to each generation specifically. Since the ordinary genetics terms P, $F_1$, $F_2$, etc., are inadequate for this type of life cycle which includes several modes of reproduction as the sexual, budding, floatoblast and sessoblast methods it is suggested that the following new terms be used for a part of the life cycle and that others be introduced later as urgent need for them arises and when more is known of the life cycles of some of these forms. The floatoblasts which were begun or produced in the ponds or lakes before collection and which were brought indoors to start new colonies were designated by the symbol "$FG_1$" which means "Floatoblast Generation Number One." The immediate colonies produced by their germination were called "$FG_1$-produced colonies." When the $FG_1$-produced colonies formed a new generation of floatoblasts these new floatoblasts were called "$FG_2$" or "Floatoblast Generation Number Two." When they in turn germinated and gave rise to colonies the latter were called "$FG_2$-produced colonies." A new generation of floatoblasts arising from the $FG_2$-produced colonies would be called "$FG_3$," etc.

### *$FG_2$ Development*

The floatoblasts used in Groups or Sets of P, Q, R, S, T and U were all $FG_1$. The colonies some of them elaborated and on which Tables I to V are based, were $FG_1$-produced colonies. In time, on the funiculi of their polypides appeared $FG_2$ (floatoblast) Anlagen, rudiments or "germ" (Fig. 4). In this species, *H. punctata*, the usual number of rudiments was two per funiculus although the number occasionally was greater, other times less. In a recently examined *Plumatella repens* var. *fruticosa* colony the number of statoblast rudiments on one funiculus was fourteen, the most mature one being closest to the caecal end of the digestive tract and the youngest one being most distally located with respect to the tract.

The *H. punctata* floatoblast rudiments appeared on funiculi of evaginated buds and also on those of buds which were so small and immature that even their tentacles were not yet clearly defined nor evaginated. The rudiments were lumps of very small size on the funiculus when first noted.

The first $FG_2$ (floatoblast) rudiments were evident 13 days after germination of Colony R-4e, 16 days after germination of Colony R-4b and 24 days after germination of Colony R-4d, or, if time is reckoned from the date of $FG_1$ immersion rather than from colony germination then the floatoblast rudiments ($FG_2$) were first noted 21 days after immersion in Colony R-4b, 22 days after immersion in Colony R-4e and 25 days in Colony R-4d. No $FG_2$ rudiments appeared in Colony R-4a even by the 32nd day after immersion of $FG_1$, (or by the 28th day after germination of the colony), although by that time the colony had produced twelve

polypides. The number of polypides which had evaginated by the time the first floatoblast rudiments made their appearance was three in Colony R-4e, four in Colony R-4d and seventeen polypides in Colony R-4b. These floatoblast-Anlagen appearance and formation figures for *H. punctata* are similar to those given by the writer for *Lophopodella carteri* (Study VII, p. 194, 13 to 59 days after germination) and somewhat larger than figures given by Dr. Marcus for *Stolella evelinae*. Marcus stated (1941, p. 150) that, "Statoblasts (of *S. evelinae*) occur as early as in the funiculus of the second or third individual of a statoblast colony," and that "the formation of a statoblast takes 8 days."

Sometimes the development of $FG_2$ (floatoblast) rudiments proceeded to completion. Other times degenerative processes set in and the rudiments failed to continue their development. Roughly speaking, floatoblasts seemed to be produced in series but it was difficult to tell when one series ended and another began, particularly since colony degeneration set in now and then because of improper food or other condition and because new statoblast rudiments did not appear simultaneously over the colony. With the alleviation of some of these conditions the life of the colony was prolonged and new floatoblast rudiments got a chance to form while frequently some of the previously begun statoblast rudiments disappeared. Therefore it was best to place the data in Table IV, rather than to attempt to number the various series of statoblasts which were produced. For example, Colony R-4b produced varying numbers of statoblasts whose rudiments were first noted on the following days: 21, 39, 67, 87, 91 and 135 days after immersion of the $FG_1$.

When colonies containing developing statoblasts in advanced stages of maturity or brownness fragmented, some fragments eventually lost all their polypides through degeneration and became mere coenoecial bags containing floatoblasts while other fragments continued their development after recovery. The polypide-less sacs gradually decreased in size but the brown statoblasts remained inside until dissected out with needles. One such polypide-less sac contained an immature floatoblast which continued its development to a slight extent but had almost no float although its capsule was pretty well developed.

In Colony R-4b, the first two floatoblasts of $FG_2$ were released 12 days after their rudiments were first noticed in the colony. A third statoblast was released 14 days after first being noticed and forty statoblasts were released between 26 and 36 days after first notice of their rudiments. Table IV, last column, shows some of this data and more also. The floatoblasts when released sank to the bottom of the dish.

A total of 92 floatoblasts ($FG_2$) were released by Colony R-4b, mostly normally but a few with the aid of dissecting needles. These floatoblasts were divided into several groups for further study. One group was set out immediately after release, to germinate. Another group was dried and stored dry in vials at room temperature. A third group was dried and stored thus in the refrigerator. A fourth group was stored in a small quantity of tap water in the refrigerator. All but three floatoblasts of the first $FG_2$ group of twenty-one which had been set out to germinate immediately after release or dissection from the parent colony R-4b did hatch. Their germination occurred between 13 and 35 days (average 27.5 days) after removal from parent colony or its debris. They had been in water all the time from release from the colony and had not been subjected to chilling or drying. The other three groups have been set aside for future experiments.

### *Sperm Development*

Thus far asexual reproduction of two types, by budding and by floatoblasts, has been discussed. The early stages of sexual reproduction, particularly sperm production, were visible for a brief interval of five or six days in Colonies R-4a and R-4b respectively. Spermaries appeared as irregular, lumpy, granular masses

on the funiculus of both evaginated and as yet unevaginated buds but for some reason were not found on the funiculus of the ancestrula in the colonies under observation. After a short time the sperm masses looked rather massive and hairy, the sperms making a squirming, undulating hairy covering over a large part of the funiculus. Later still long, slender, hair-like sperms could be seen circulating about in the body cavity. This latter condition prevailed five or six days after the spermaries were first noticed. It also marked the end of the spermary as a visible mass. Sperm masses formed very speedily and were noticeably well developed in Colony R-4b by the 10th day after germination, in Colony R-4a by the 18th day after germination and in Colony R-4d before the 24th day after germination. They appeared sooner than floatoblast rudiments in these colonies.

## CONCLUSIONS

*Hyalinella punctata* floatoblasts were divided into several groups. Each group was kept under a slightly different set of conditions. The highest percentage of germinations, 71.1%, occurred in the R Group of 135 floatoblasts which had been placed in a refrigerator in liquid. After a while there they had become dry and were stored dry at the same low temperatures (7–13.5° C.) from 244 to 863 days. The R Group furnished the longest lived colony of any other group. Groups S floatoblasts which had been stored wet in a refrigerator for 540 days, after a long period of freedom in water at room temperature showed a slightly higher percentage of germinations, 28.5%, than Group T floatoblasts which had been stored in water at room temperature for 327 days (25% germinations).

Considering the difference in age of the statoblasts this percentage difference assumes more value. S and T Groups both showed higher percentages of germination than did Group U, 2.2%, which consisted of floatoblasts which had remained free indoors in water for a long time at room temperature, then were dried and stored in a refrigerator at from 7° to 13.5° C. for 306 to 540 days. The poorest showing as regards germinations was made by Groups P and V, neither of which produced any. Floatoblasts of both groups had been dried a few days after collection. Those in Group P were then stored dry at room temperature from 289 to 1412 days while those of Group V were stored dry in the refrigerator for 840 days. The Q Group, consisting of floatoblasts which had remained indoors at room temperature throughout a long interval of freedom in liquid followed by a 550 day interval in the dry condition, gave rather odd results. Although there was a 50% germination (?) or more precisely a splitting apart of the valves of half of the statoblasts no polypides evaginated or were visible. Therefore it was hard to compare this group with the others.

Based upon the foregoing results it would seem that the least satisfactory method of keeping *Hyalinella punctata* floatoblasts alive for future germinations would be to dry them soon after gathering then store them dry at room temperature. This method, however, proved very satisfactory for *Lophopodella carteri* some of whose floatoblasts retained their viability for after four and one-quarter years in the dry state at room temperature (Study XI, p. 318). Unfortunately, no *Lophopodella carteri* floatoblasts were stored in the refrigerator for any considerable length of time so that a comparison between viability retention through drying and through chilling can not be made at the present moment for that hardy species. A more satisfactory method for keeping *H. punctata* statoblasts viable was to place them for a few weeks in a refrigerator, allowing them to become dry there and to remain there at temperatures between 7° and 13.5° C. until ready for use. *Hyalinella punctata* statoblasts so treated remained viable 867 days after collection and produced good colonies. The upper limit of viability has not been reached for *H. punctata* floatoblasts so treated.

To begin germination experiments, floatoblasts were removed from their storage places and placed in shallow dishes partly filled with tap water and there left until germination occurred.

After the floatoblasts germinated the resultant colonies were fed by various foods: bacteria, algae, Protozoa and organic debris from various food products. Addition of small quantities of fresh yeast to the Bryozoan culture proved very beneficial, both as a growth stimulant and as a means of helping to prevent degenerative processes from coming to a too speedy culmination.

Although rearing conditions were not always most satisfactory, nor was the food always the most suitable, nor the cultures always free from Bryozoan enemies, nor the colonies always hardy, it was still possible, because of a great deal of care and attention, to keep some of the *H. punctata* colonies and fragments alive 176 days after floatoblast germination, a longer time than has been recorded for any other fresh-water Bryozoan species to date.

Out of 323 variously treated *Hyalinella punctata* floatoblasts which were immersed in water for purposes of germination, 124 hatched in one to twenty-five days after immersion. Of these 124 germinations, 111 evaginated first polypides or ancestrulae in one to eight days after germination, thirty-nine evaginated second polypides, sixteen evaginated third polypides, eleven evaginated fourth polypides, ten protruded fifth polypides, eight evaginated sixth, seventh and eighth polypides, seven evaginated ninth zooids, six evaginated tenth zooids, five evaginated up to twelve polypides, four evaginated up to sixteen polypides, three up to the eighteenth polypide, two evaginated nineteenth polypides and one, the long-lived colony and its fragments produced and evaginated more than 500 polypides. Because of the colony's tendency to fragment or divide into several smaller colonies not all the $500^{+}$ polypides derived from the germination of one floatoblast were in one mass but were distributed among several colony fragments. At one time there were as many as twenty-eight colony fragments from the one statoblast and at another time there were as many as 159 polypides evaginated in a single fragment. The time interval for evagination of each series of polypides, first, second, third, etc., is given in detail in the "Successive Polypides" section and Tables II and III.

Extensive study of polypide degeneration was not made except in the case of the ancestrula. The ancestrulae, which were generally the first to degenerate, began to do so from three to twenty-one days after floatoblast germination. Data on degeneration of some successive polypides is also given in the text of the article.

Because of the long life span, studies on (a) the rate of polypide addition, degeneration, formation of sperms and of a new floatoblast generation and (b) the manner of growth, behavior and other bodily or colonial processes were possible. The general developmental and colonial processes were similar to those of *Lophopodella carteri* although the rate or time interval for the various processes differed somewhat.

Sperm masses appeared early, from ten to twenty-four days after statoblast germination and lasted a very short time, for five or six days. A new generation of floatoblasts was first noticed developing from sixteen to twenty-four days after germination of their parent floatoblasts. Some of these new floatoblasts matured and were released from the colony as early as twelve days after their Anlagen were first observed while others took longer, some as long as thirty-six days. The total number of new generation ($FG_2$) floatoblasts obtained from the longest lived colony was 92, some of which were put aside for future germinations and some of which germinated between thirteen and thirty-five days after release from the parent colony.

The new symbols and expressions $FG_1$, $FG_1$-produced colonies, $FG_2$, $FG_2$-produced colonies, $FG_3$, etc., were introduced to distinguish between the various generations of statoblasts and colonies derived from their germination. The $FG_1$ stands for the First Floatoblast Generation (that used to start this particular

experiment). The colonies derived from it were called $FG_1$-produced colonies. They in turn produced a new floatoblast generation known as $FG_2$. The colonies which germinated from this new generation of floatoblasts were called $FG_2$-produced colonies. These in turn gave rise to a third generation of floatoblasts known as $FG_3$, etc. The terms will simplify reference to any particular generation.

## SUMMARY

1. *Hyalinella punctata* floatoblasts, collected from two localities, New Rochelle, N. Y., and Westtown, Pa., were used in these experiments.

2. The "age" of the 323 floatoblasts used, counting from the time of collection of the mature floatoblast to the time of its immersion in shallow dishes partly filled with tap water for purposes of germination, ranged from 248 days to 1420 days. Germinations were obtained from those whose "age" ranged from 248 to 877 days but not from those of 1420 days. The failure of the last to germinate was due perhaps not so much to age as to the method of storing them (dried and at room temperature). Those which did germinate had been stored under slightly different conditions,—some involving chilling, some storing while wet, some drying after long wet intervals, etc. For greater details see Table I.

3. Out of the 323 variously treated floatoblasts, 124 hatched, 111 of these evaginated ancestrulae, 39 evaginated second polypides and a progressively smaller number evaginated larger numbers of successive polypides up to the nineteenth polypide. From the twentieth polypide on, only one colony (which fragmented many times) continued its development.

4. The longest lived colony produced more than 500 evaginated polypides, at one short period was divided into as many as twenty-eight separate fragments and produced 92 mature floatoblasts, some of which hatched and some of which were stored for future experiments. It also produced sperms. Some of its fragments lived till the 176th day after the germination of the original floatoblasts or till the 181st day after their immersion. This length of time is a record for any statoblast-derived Bryozoan colony kept under laboratory conditions.

5. Because of these germinations and the progress of some of the colonies it was possible to obtain definite information on the rate of formation and degeneration of polypides, the time of appearance and rate of formation of new statoblasts and sperms, the habits of colonies and individuals, the manner of rearing and keeping colonies and statoblasts. Much of this data is embodied in the several tables of the article.

6. Several new symbols and terms are introduced to distinguish the various generations of floatoblasts and the colonies derived from them: $FG_1$, $FG_1$-produced colonies, $FG_2$, $FG_2$-produced colonies, $FG_3$, etc.

7. Included also is a small glossary of terms pertaining to fresh-water Bryozoa.

## GLOSSARY OF FRESH-WATER BRYOZOAN TERMS

ANCESTRULA—This is the primary or first individual of a colony. It comes from between the valves of a germinating statoblast, to elaborate a colony. In marine Bryozoa it has a slightly different origin.

BUD—This is an individual produced by the proliferation of cells in a definite part of the body wall of an individual of the colony.

CAPSULE—This is the darker colored, usually brown, part of a statoblast enclosing the germinative material.

COENOECIUM—This is the name given the common dermal system of a colony by Allman and includes the ectocyst and endocyst.

DUPLICATURE—This is the fold of body wall around the base of the tentacle sheath or introvert when the polypide is evaginated. It is also known as the sigmoid fold and as the invaginated fold.

ECTOCYST—This is the outer of the "two" layers of the body wall. It may be one or more of the following: thin, thick, delicate, firm, soft, swollen, gelatinous, transparent or hard, crusty, deeply colored (yellow to brown or reddish brown) depending upon the genus and species.

ENDOCYST—This is the inner of the "two" layers of the body wall. In reality it is not a single layer but may consist of several layers as shown by Dr. Borg and others.
EVAGINATION—This is the act of protrusion of the introvert or tentacular crown and tentacular sheath from the coenoecium.
$FG_1$—The first or parent generation of floatoblasts used in a set of germination experiments. Defined in present Study.
$FG_1$-PRODUCED COLONIES—These are the colonies derived from the germination of the $FG_1$ (floatoblasts).
$FG_2$—Floatoblast generation No. 2, developed from the $FG_1$-produced colonies.
$FG_2$-PRODUCED COLONIES—Colonies derived from the germination of $FG_2$ (floatoblasts).
$FG_3$—Floatoblast generation No. 3, developed from the $FG_2$-produced colonies.
FLOAT—It is that part of the statoblast known as the "cellular" annulus. It covers part of the capsule and usually extends beyond it.
FLOATOBLAST—This is elsewhere known in literature as a free or floating type of statoblast which does not possess spines or barbs or hooked processes. Defined more fully in Study XIV.
FUNICULUS—This is the cord of tissue attaching the digestive tract to the body wall. From it develop the floatoblasts of the Phylactolaematous Bryozoa.
GERMINATION—This is the splitting apart of the two valves of a floatoblast to permit the further growth of the germinative material or preformed polypides which are within the capsule.
INTROVERT—That part of the polypide which is evaginated and which begins in the region of the duplicature. It includes the tentacular crown and tentacular sheath.
INVAGINATION—The withdrawal, either temporary or permanent, of the polypide into the body cavity.
LARVA—The sexually produced, free-swimming ciliated stage in the life cycle.
LOPHOPHORE—This is a horseshoe-shaped or circular ridge on which are borne the tentacles.
POLYPIDE—Prouho (in Borg, p. 190) defines it as a single word or unit comprising the organs of digestion and muscular activity of an individual. It refers to the soft parts of an individual and includes the tentacular crown.
RETRACTORS—The large bundles of muscles attached to the lophophore and to the upper part of the digestive tract which are concerned with pulling the polypide in during invagination.
SESSOBLAST—A statoblast which was formerly called sessile, fixed or attached. Term defined in Study XIV.
SPINOBLAST—This is a floating or free statoblast which is provided with spines, barbs or hooked processes. It is more fully defined in Study XIV.
STATOBLAST—A hard chitin-walled body or gemma of various but characteristic shapes produced in large numbers by many fresh-water Bryozoa. It contains within its capsule germinative material which is capable of producing a colony when conditions are suitable for its germination.
TENTACULAR CROWN—Consists of the lophophore and its tentacles.
TENTACULAR SHEATH—That part of the introvert which encloses the tentacles when the polypide is invaginated.
ZOARIUM—Refers to the colony of the Bryozoan.
ZOOECIUM—The external skeleton or remains of the firmest part of the body wall of an individual of a colony.
ZOOID or ZOID—Any one of the living individuals of a colony.

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# A NEW SPECIES OF AGRILUS FROM KENTUCKY

## (Buprestidae: Coleoptera)

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**Agrilus cladrastis** n. sp.

*Male.*—Size and form of *A. celti* Knull, uniformly dark bronze; legs with greenish lustre; head metallic green on front.

Head convex, granulose on front, finely longitudinally rugose on occiput, clothed on lower half with dense white pubescence; antennae extending beyond middle of prothorax when laid along side, serrate from fifth joint.

Pronotum wider than long, narrower at base than at apex; sides subparallel in front, strongly narrowed basally; when viewed from side, marginal and submarginal carinae separated in front, united near base; anterior margin sinuate with broad median lobe; basal margin transversely sinuate; disk convex, with two slight median depressions, a deep lateral depression each side near margin, prehumeral carina on each side well marked. Scutellum transversely carinate.

Elytra wider than base of pronotum; sides subparallel basally, constricted about middle, widened back of middle, then narrowed to rounded serrulate apices; disk feebly longitudinally depressed, sutural margin elevated posteriorly, a broad basal depression on each elytron; surface imbricate.

Abdomen beneath finely punctate, pubescence inconspicuous, first two segments flattened in middle, slightly concave. Prosternal lobe broadly rounded in front, broadly emarginate at middle. Posterior coxa with hind margin broadly emarginate, outer angle rectangular. Anterior and middle tibiae mucronate on inner margin at apex. Posterior tarsi shorter than tibiae. Tarsal claws similar on all feet, cleft, outer tooth long, acute; inner tooth broad, very short.

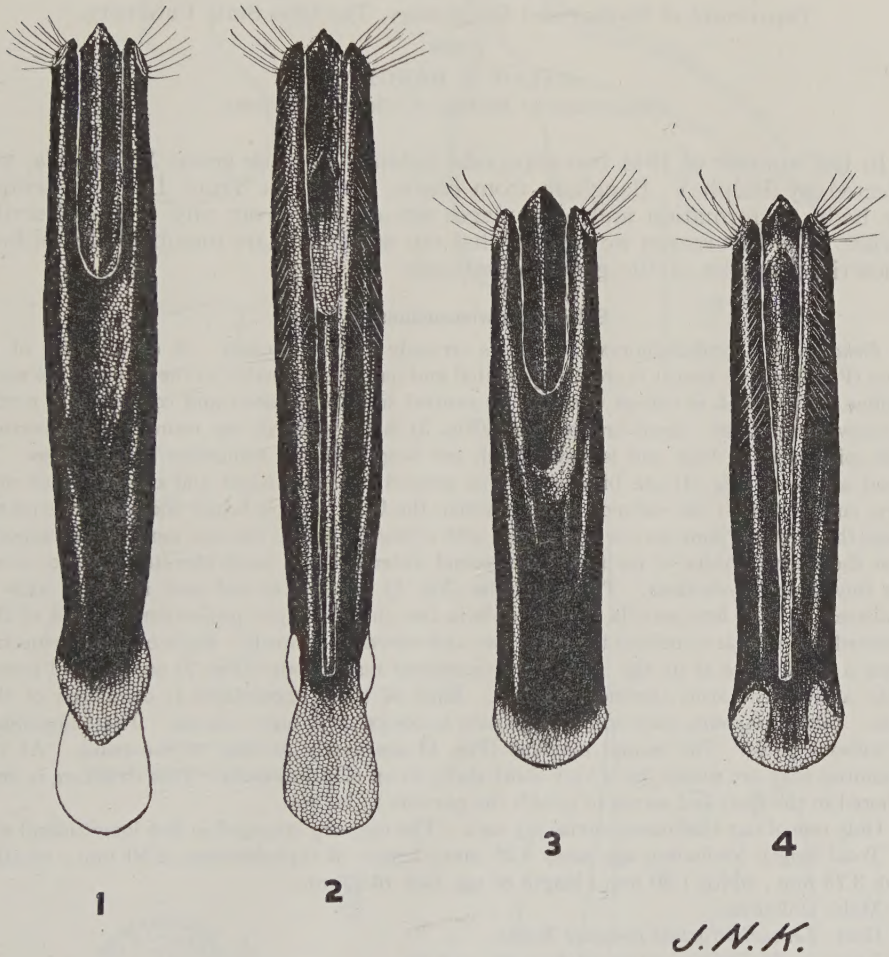
Length 4.6; width 1.2 mm.

*Female.*—Differs from male by not having tibiae mucronate; first two abdominal segments convex at middle.

Described from a series collected on the foliage of yellow wood (*Cladrastis lutea* (Mich.)), at Lexington, Ky., April 15, 1941, by L. H. Townsend. Holotype male, allotype and paratypes in collection of writer. Paratypes in collections of University of Kentucky and The Ohio State University.

This species runs to *A. celti* Knull in Fisher's key.[1] The males lack the brilliant viridescence of many *celti* and the median ventral line of pubescence extending from prosternum to second abdominal segment of *celti* is lacking. The male genitalia are different also.

[1]W. S. Fisher, 1928. U. S. Nat. Mus. Bull. 145: 1–347.




EXPLANATION OF PLATE

Male genitalia of *Agrilus;* figures 1 and 3 represent dorsal surfaces; figures 2 and 4 are ventral views.

Figures 1 and 2, *celti* Knull; 3 and 4, *cladrastis* Knull.

# A COPEPOD PARASITE OF THE CISCO FROM TROUT LAKE, WISCONSIN

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In the summer of 1944 two copepods, belonging to the genus *Salmincola*, were collected by Ralph V. Bangham from ciscos, caught in Trout Lake, Wisconsin. The body conformation and appendages are distinct from any of the described species. For this reason we suggest that our specimens are members of a hitherto undescribed species of the genus *Salmincola*.

**Salmincola wisconsinensis** n. sp.

*Female.*—The cephalothorax (Fig. 1) is strongly arched dorsally. A dorsal view of this region (Fig. 2) shows that it is greatly elongated and narrowed anterior to the bases of the second maxillae. The trunk is curved dorsally, its ventral surface flattened and containing a number of transverse grooves. Each first antenna (Fig. 3) is two-jointed, the terminal joint carries a small spine near its base and is tipped with one large and two somewhat smaller ones. The second antennae (Fig. 4) are biramose. The exopod is knob-shaped and covered with many sharp, curved spines; the endopod is two jointed, the basal joint is broad and also covered with spines, the terminal joint narrow and armed with a broad spine on the side next to the exopodite. Upon the lateral border of each of these second antennae is a small elevated surface covered with tiny, blunt projections. The mandibles (Fig. 5) are slender and each is armed with five distal teeth. Each first maxilla (Fig. 6) ends in two thumb-shaped projections, a third of these is located some distance behind the other two and curves backwards. Each of these projections carries a small spine at its tip. The very prominent maxillipedes (Fig. 7) are set well forward of the attachment arms (second maxillae). Each of these appendages is composed of three joints. Like *S. inermis*, they do not terminate in hooks, as in other species. These appendages are without palps. The second maxillae (Fig. 1) are nearly as long as the trunk. At their extremities they are joined, by a very short stalk, to an elliptical bulla. This structure is firmly anchored in the flesh and serves to attach the parasite to its host.

Only one of our specimens carries egg sacs. The eggs are arranged in five longitudinal rows.

Total length (excluding egg sacs) 6.25 mm.; length of cephalothorax 2.50 mm.; length of trunk 3.75 mm., width 1.20 mm.; length of egg sacs 10.32 mm.

Male: Unknown.

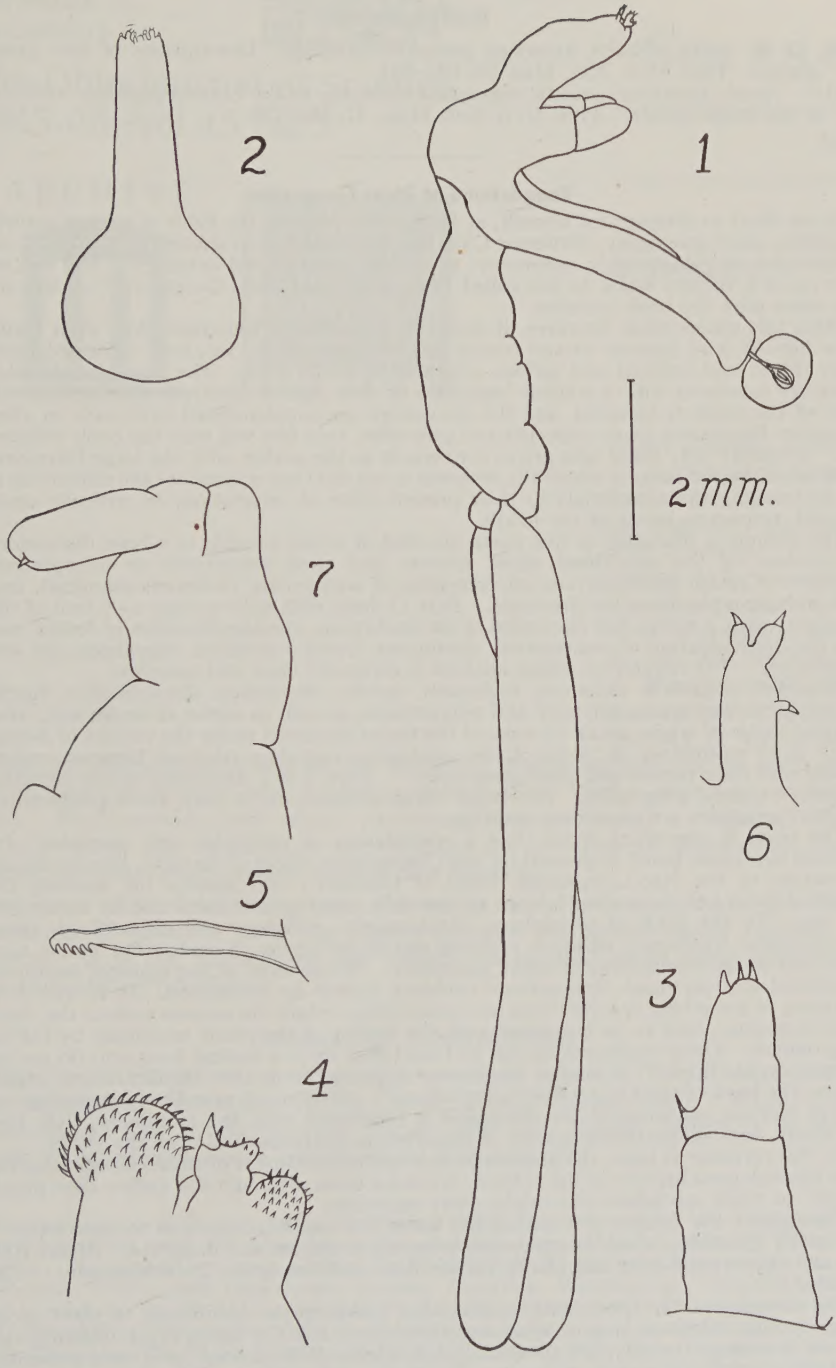
Host: *Leucicthys artedi clemensi* Koelz.

Location: Upon the surface of the body and fins.

Holotype: U. S. Nat. Mu.s Catalog No. 81553.

## DISCUSSION

The affinity of the species described above is with *S. inermis* (Wilson) which is also a parasite of the cisco. However, it can be readily differentiated from Wilson's species by the marked differences in the morphology of the antennae, narrow and pointed head, and an entirely different shaped bulla. The egg sacs are not turned forward along the sides of the body as in *S. inermis* but are carried behind the trunk. The two species differ further in their choice of a location upon the host. *S. inermis* is a parasite of the gill cavity. Its umbrella-shaped bulla is held among the gill filaments, while in *S. wisconsinensis* this structure is firmly embedded upon the surface of the body and fins.




All figures drawn by the aid of a camera lucida from glycerin mounts.

Fig. 1. Lateral view of female. Fig. 2. Dorsal view of cephalothorax. Fig. 3. First antenna. Fig. 4. Second antenna. Fig. 5. Mandible. Fig. 6. First maxilla. Fig. 7. Anterior view of a maxillipede.

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**Foundations of Plant Geography**

In an effort to discover for himself, at least, what some of the fields of science contribute to interpretive plant geography, Professor Cain has attempted to evaluate the materials, concepts and principles of paleontology, taxonomy, evolution, genetics and cytology. The end result of the survey is a volume which he has called "Foundations of Plant Geography"—a title implying rather more than the book contains.

With this observation, however, it must be immediately remarked that what Cain found out for himself is of interest to and constitutes necessary study for, both informed teachers of ecology (plant and animal) and serious advanced students alike. The factual materials in this volume are presented with a studied broadness of view and an apparent thoroughness of examination of the subjects included, and the discussions are supplemented with such an abundance of examples illustrating many concepts and principles, that few will read the book without much profit. Probably only those who are as conversant as the author with the large literature of the several fields treated (among whom the reviewer is not one) can appreciate the difficulties encountered in bringing these materials to their present state of integration, or critically review the work with respect to many of its details.

The volume is presented in five parts, the first of which consists of a brief discussion of the inter-relations of the specialized plant sciences and their importance to interpretive plant geography. Certain previously-stated principles of ecology are reviewed, examined, and illustrated with examples from the literature. Part II deals with paleoecology as a tool of the plant geographer, with a rather full discussion of its limitations, the identification of fossils, and their use in the determination of composition, dominance, living conditions, migrations and evolution of vegetation. The chapter on pollen analysis is especially clear and complete.

Dispersal, migration endemics, endemism, species senescence, discontinuous distribution, vicarious forms and areas, polytopy and polyphylesis, as well as center of origin and criteria for indicating center of origin, make up some of the topics discussed under the subject of Aerography. Part IV is an assemblage of "some of the conclusions regarding relations between evolutionary processes and their results and plant geography." Part V is a discussion of the significance of polyploidy in plant geography. The latter three sections are in part, more philosophical and illustrative examples are sometimes wanting.

The book is something more than a compilation of principles and examples. It is an attempted synthesis, based in general on the Clementsian school of thought, despite the author's renunciation of the Neo-Lamarckian views of Clements; and despite his warning that the organismal nature of vegetation is open to question, assumptions discarded by many ecologists years ago. To the fields of physiology, physiography, pedology and climatology, the author gives no special treatment, although utilizing certain principles of each. For many, these too, form a part of the foundations of plant geography. While most of the material included seems fundamental and pertinent, one curious tendency should be mentioned. It is with hope and expectation of something concrete that one approaches certain discussions such as the chapter on species senescence, only to be impressed with the futility of the whole treatment by the author's closing remark: "Other explanations can be found that have a factual basis and do not have an anthropomorphic taint." A similar impression is gained from the classification of plant propagules on the basis of their migration "adaptations." After much grandiose terminology and the listing of obvious advantages, the discussion is concluded with the statement: "On the other hand, *Carex*, with no particular means of dispersal, is distributed all over the world."

To this reviewer at least, the absence of any special treatment of microclimates, barriers and certain physiological aspects of the subject, is conspicuous, although the author does plead "lack of space" to those specialists who might decry omissions.

Throughout the volume the author has eased the reader's approach to new topics with a statement of principles, which he proceedes to examine, discuss and illustrate. About 100 maps, tables and charts emphasize and clarify certain data and concepts. A bibliography of 720 titles is included.

The diversity of the specialized sciences that make up the foundation of plant geography, their many inter-relations, their overlapping viewpoints, and the difficulty of obtaining complete field data in ecological study, are adequately indicated. With a need for a lucid presentation of the relations among these anastomosing disciplines and methods—with such a need for this pioneer job in particular—it is regrettable that so much of the terminology is unnecessary, and some pedantic. Nevertheless, it is a much-needed, useful and stimulating work—*John N. Wolfe.*

**Foundations of Plant Geography,** by Stanley A. Cain. Harper and Brothers, New York. 1944. $5.00.